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Studies on the Anemia of Tumor-Bearing Animals. III. Localization of Erythrocyte Iron Within the Tumor [1]

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Summary

When erythrocytes tagged with Cr^{51} were mixed with erythrocytes labeled with Fe^{59}, both isotopes were distributed in the same pattern in Lymphosarcoma R2788 of the rat. Most of the radioisotope was deposited in hemorrhagic areas, almost none in firm, viable tumor, and an intermediate amount in the necrotic areas. Most of the iron found within the tumor was shown to have been derived from the erythrocytes. Microscopic examination of stained and incinerated sections of Lymphosarcoma R2788 showed a major portion of the iron to be present as deposits of hemosiderin in macrophages surrounding the hemorrhagic areas, with little being detectable within the tumor cells. Other tumors had widely varying patterns of vascular injury. The relationship of the anemia in the host to the vascular lesions in the tumor is discussed.—**J. Nat. Cancer Inst. 22: 877–885, 1959.**

Earlier studies (*1*, *2*) demonstrated that in anemic rats and mice bearing different types of tumors, a process of erythrocyte destruction is important in the pathogenesis of the anemia. It has been shown that this destructive process is due largely to the random loss of intact erythrocytes from the blood stream by their deposition and destruction within the tumor. The process of erythrocyte destruction was most marked in animals bearing Lymphosarcoma R2788. The marked fluctuation in the rate at which erythrocytes labeled with Fe^{59} disappeared from the blood stream of rats bearing this tumor was consistent with a series of separate hemorrhages, presumably resulting from vascular lesions within the tumor. Gross examination of the lymphosarcomas, however, revealed only small areas of hemorrhage, and, on the basis of a hemorrhagic process, it was difficult to account for the daily loss of as much as 10 percent of the circulating erythrocytes into the tumor.

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The present paper deals with a more detailed study of the localization of the iron of labeled erythrocytes within the tumor, and with an attempt to correlate the isotope studies with the morphologic features of the tumor. The experiments described showed that the Fe^{59} of labeled erythrocytes was localized in the hemorrhagic areas of the tumor, and that very little iron was found in the firm, white nodules of viable tumor. Microincinerated sections of the tumor showed that much of the iron was contained within macrophages in the connective-tissue stroma surrounding the hemorrhagic areas. The morphologic characteristics of other tumors used in these studies (*1*, *2*) were also correlated with the rate of destruction of erythrocytes and with the degree of anemia and cachexia of the host.

Materials and Methods

The method for preparation and transfusion of labeled erythrocytes and the general techniques employed have been described previously (*1*). For histologic study, tissue slices excised from various portions of each tumor were fixed in a solution containing 9 volumes of absolute alcohol and 1 volume of 37.4 percent formaldehyde. The sections were cut 6 μ thick, and every third section was stained with hematoxylin and eosin. Adjacent sections were examined for iron by the Prussian-blue reaction and by microincineration,[5] with Scott's (*3*) modification of the Policard microincinerator (*4*). Microincineration was accomplished by passing a glass slide carrying the paraffined section, at a controlled rate of speed, through a ventilated open quartz tube, thus exposing the tissue consecutively to temperatures of 80, 250, and 600° C. for 60 minutes. By this technique nearly all the organic substances were removed, and the mineral constituents of tissues and cells were reduced to their inorganic state in essentially the same topographic distribution as existed in the fixed-tissue section. In microincinerated sections under dark-field illumination, the iron of ferritin and hemosiderin appears as bright orange-red deposits of iron oxide, whereas iron of the heme proteins appears as small yellow particles. The oxide of iron can thereby be distinguished readily from the flat-white ash of the oxides of calcium and magnesium and from the blue ash of the oxides of sodium and potassium. The oxides of silicon form yellow-gold ash, but may be distinguished by a high degree of birefringence not characteristic of the ash of the other mineral elements demonstrable in tissues by microincineration (*3–6*).

Experiments

Localization of Noncirculating Iron and Fe^{59} Within Lymphosarcoma R2788

A study was carried out to correlate the distribution of iron and Fe^{59} with the gross appearance of the tumor. To do this, 0.50 ml. of labeled erythrocytes containing 0.38 μc of Fe^{59} was injected into three M520 × (A

[5] We wish to thank Frank G. Miner for preparing the histologic sections, and for carrying out the microincineration.

× C)/9935 male rats into which Lymphosarcoma R2788 had been implanted subcutaneously 22 days previously. After 7 days, when the level of circulating Fe^{59} had fallen to about 55 percent, the animals received an injection of 0.25 ml. of erythrocytes tagged with 1.7 μc of Cr^{51} to provide a label for the circulating blood. After 30 minutes, the animals were killed and the tumors sliced into sections 3 to 5 mm. thick. In its gross appearance, most of the tumor tissue was white with relatively little evidence of hemorrhage. Areas of hemorrhage were found, however, at the border between the tumor and its capsule and extending into the connective-tissue trabeculae between the tumor nodules (fig. 1). A few nodules of tumor tissue showed necrotic changes in localized areas, which were usually hemorrhagic. Typical areas of hemorrhage, of necrosis, and of white, firm, viable tumor tissue were cut from the tumor, placed in tared counting vials, weighed, and differentially counted (*1*) to ascertain the Fe^{59} and Cr^{51} content of the slices. The tissues were then transferred to digestion tubes for the determination of their iron content. The Cr^{51} content of each tissue provided a means of estimating the Fe^{59} and iron in the tissue that arose from the circulating blood (*1*). The values presented in table 1 represent the noncirculating Fe^{59} and the noncirculating

TABLE 1.—*Distribution of noncirculating iron and noncirculating erythrocyte radioiron in Lymphosarcoma R2788*

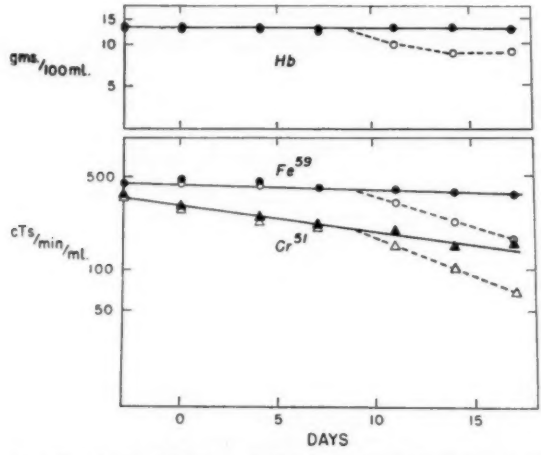
Area of tumor	Iron (*μg./gm.*)	Radioiron (*cts./min./gm.*)
White, viable tissue	58, 86, 77	19, 19, 28
Hemorrhagic tissue	205, 141, 344	179, 95, 161
Necrotic tissue	106, 164, 125	19, 82, 95

iron content of the representative areas of the tumor. It will be seen that both Fe^{59} and iron were confined chiefly to the hemorrhagic areas, though the necrotic tissue contained moderate amounts of iron where earlier hemorrhage had occurred. The firm, white tumor nodules contained very little Fe^{59} or iron. Since the viable tumor tissue contained so little iron, it seemed highly unlikely that the hemoglobin served as a source of protein for the nutrition of tumor cells (*2*). It was apparent that the erythrocytes were being destroyed in hemorrhagic lesions in the capsule and in the trabeculae between the tumor nodules.

The Fate of Fe^{59}- and Cr^{51}-Labeled Cells Injected Simultaneously

To compare the fate of erythrocytes labeled with Fe^{59} and Cr^{51} within the tumor, labeled erythrocytes containing 0.14 μc of Fe^{59} were mixed with tagged cells containing 1.0 μc of Cr^{51}; then 0.5 ml. of cells containing 63 mg. of hemoglobin was injected into 6 male rats of the M520 × (A × C)/9935 strain. Three days later, implants of Lymphosarcoma R2788 were inoculated subcutaneously into 4 animals, on each side of the posterior lumbar region. The levels of the 2 isotopes and of hemoglobin in the

blood were determined on samples taken from the caudal vein at 3- to 4-day intervals after transplantation of the tumor, and these data are shown in text-figure 1. In these animals, the hemoglobin level provided a measure of the host's own cells; the Fe^{59} levels represented the fate of newly formed erythrocytes into which the radioiron had been recently incorporated, and the Cr^{51} levels represented the fate of red blood cells of all ages that had been randomly labeled by the radiochromate technique. The concentration of Cr^{51} fell continuously because of the senescent destruction of the older radiochromate-tagged cells, and, presumably, also because of the slow elution of Cr^{51} from the red cells, as described by Ebaugh *et al.* (7).



TEXT-FIGURE 1.—Survival of Fe^{59}- and Cr^{51}-tagged erythrocytes transfused simultaneously into rats bearing Lymphosarcoma R2788. *Solid symbols* represent normal animals; *open symbols* represent tumor-bearing animals.

In the tumor-bearing animals, the levels of hemoglobin and of both radioisotopes simultaneously fell below those of the normal animals on about the 9th day following implantation of the tumor. The fact that the concentrations of the two isotopes fell off in parallel fashion indicates that both old and young cells disappeared at the same rate. The hemoglobin concentration leveled off after several days, presumably the result of compensatory synthesis of new red blood cells to replace those being lost by hemorrhage.

On the 17th day following implantation of Lymphosarcoma R2788, the rats were killed and the tumors removed and sliced into sections 3 to 4 mm. thick. Alternate slices were fixed in alcohol-formalin for histologic studies. From the intervening slices representative areas of hemorrhagic, necrotic, and firm, white, viable tumor tissue were selected for measurement of Fe^{59}, Cr^{51}, and iron, as described in the previous section. In this experiment, since both Fe^{59}- and Cr^{51}-labeled cells had been injected, no

label was available for the circulating blood. Therefore the data presented in table 2 represent the total values for the tissue instead of the values for noncirculating isotope and iron. The distribution of both radioisotopes and of the iron was similar to that of noncirculating iron and Fe^{59} described in the previous section, with low values being found in the firm, white, viable tumor tissues, while the markedly hemorrhagic areas had the highest values. The moderately hemorrhagic and necrotic areas had intermediate values. Since radiochromate provided a label for the protein moiety of hemoglobin, and since the distribution of Fe^{59} and Cr^{51} was similar in the various areas of the tumor, the data presented constitute additional evidence that the iron and protein moieties of hemoglobin were deposited together within the tumor (*2*).

TABLE 2.—*Distribution of radioisotopes from labeled red blood cells within Lymphosarcoma R2788*

Type of tumor tissue	Fe^{59} (*cpm/gm.*)	Cr^{51} (*cpm/gm.*)	Total Fe (*μg./gm.*)	Estimated Fe from red cells (*μg./gm.*)
Firm, white, viable tissue	18	3.6	33	20
Moderately hemorrhagic tissue	63	22	92	69
Markedly hemorrhagic tissue	113	31	159	125
Necrotic tissue	74	24	147	82

From the average ratio of Fe^{59} to hemoglobin concentration within the circulating blood, one can estimate the amount of hemoglobin iron which would have had to enter the various areas of the tumor to account for the amount of Fe^{59} found there. This is presented in table 2 as *estimated iron from red cells*, and, of course, represents circulating iron as well as that lost by hemorrhage into the tissues. It is clear that the iron estimated to have arisen from red cells constitutes the major portion of the iron within the tumor.

Pathology of the Tumors

Transplanted tumors.—On examination of histologic preparations, the nodules of Lymphosarcoma R2788 that appeared nearly white by gross examination showed limited evidence of hemorrhage (fig. 1). A band of hemorrhage between the tumor nodules and the capsule extended along the trabeculae between the tumor nodules. In the connective tissue adjacent to the hemorrhagic area, there were numerous macrophages filled with hemosiderin (fig. 2). Another section taken from the center of the same tumor (fig. 3) showed tumor tissue, a triangular area of hemorrhage, and a connective-tissue trabecula, with many macrophages filled with hemosiderin. A microincinerated section immediately adjacent to that shown in figure 3 revealed bright orange-red deposits of iron in the trabecula, which corresponded to the area containing the macrophages filled with hemosiderin (fig. 4). In contrast to the high concentration of

iron found in the trabecula, only traces of iron were detectable in the area of hemorrhage by microincineration. The ash of the viable tumor tissue contained chiefly compounds of silicon, magnesium, and calcium, with very little detectable iron. A similar distribution of iron was also found by the Prussian-blue reaction. It was apparent from these microscopic findings that a large fraction of the iron within the tumor had been stored in macrophages at the periphery of the hemorrhagic area.

In other tumors, such as Hepatoma 3683 and the Harderian gland carcinoma 2226, the more familiar pattern of central thrombosis and necrosis was predominant. The necrotic areas frequently contained large amounts of extravasated blood. Fibrosarcoma 4956 consisted largely of connective-tissue elements and extracellular fluid and only a few vessels of very small caliber were found. Although central necrosis was present, very little blood was extravasated into the necrotic area. In rats bearing this tumor, an increase in the rate at which labeled erythrocytes disappeared from the circulating blood could not be detected until the tumors had become massive and ulceration of the skin had occurred.

Spontaneous tumors.—Although every attempt was made to select transplantable tumors that had been carried in inbred strains of animals, the question arose as to whether the peripheral hemorrhage between the tumor and the tissues of the host might not have been the result of some immunologic incompatibility between the transplanted tumor and the host. To determine whether the hemorrhagic phenomenon might be present also in spontaneous malignant neoplasms, or whether it was restricted to transplantable tumors, a survey was made of histologic preparations of several spontaneous tumors, including the original neoplasms from which two tumors of the present study (*1*, *2*) were derived. Histologic sections of the original Fibrosarcoma 8971 (*8*) showed areas of hemorrhage between the tumor and the capsule similar to those found in the Lymphosarcoma R2788. The presence of large macrophages filled with hemosiderin in and around the hemorrhagic areas attested to the *antemortem* origin of the hemorrhage. A section of spontaneous hepatoma, obtained from Dr. Harold P. Morris, showed evidence of hemorrhage within the trabeculae. Adjacent areas of this hepatoma contained many macrophages filled with hemosiderin, located chiefly along the trabeculae at considerable distance from the area of hemorrhage.

Spontaneous mammary tumors of C3H mice had large sinusoidal vessels, many of which contained thrombi of various ages and states of organization which frequently led to widespread necrosis of the surrounding tumor tissue. Although evidence of hemorrhage was less marked in these tumors, the amount of blood trapped within these large thrombosed sinusoids must have been great. In this tumor, the fall in concentration of tagged cells in the circulating blood was not so much the result of extravasation of blood from the vascular tree as it was the result of marked expansion of the blood volume within the growing tumor, which served to dilute the concentration of tagged erythrocytes. Anemia, cachexia, and death developed relatively late in these animals.

Discussion

It is apparent from the present studies that the pattern of vascular lesions varies greatly from tumor to tumor, and that the gross hemorrhagic appearance of a tumor is not of itself a measure of the rate of erythrocyte destruction occurring within that tumor. In Lymphosarcoma R2788, in which hemorrhage into the connective tissue surrounding the tumor nodules is an important phenomenon, relatively little necrosis was to be found, so that macrophages could move directly into the area of hemorrhage, rapidly destroy the extravasated blood, and store the iron in the form of hemosiderin. Since the red cells were being phagocytized, comparatively little hemorrhage was observed, though large amounts of hemoglobin iron had been deposited in the area of the tumor.

On the other hand, in those tumors in which thrombosis and necrosis predominate, the hemorrhagic appearance varied greatly depending on the degree of vascularity of the tumor and the amount of blood extravasated into the necrotic area. In the tumors of the present study, this variability ranged from that of Fibrosarcoma 4956, which became necrotic without the appearance of gross hemorrhage, to that of the C3H mammary tumor, whose large thrombosed sinusoids gave it a very hemorrhagic appearance on gross examination. In animals bearing these tumors the rate of red cell destruction was very slow. In contrast to this, animals bearing Hepatoma 3683, which also had a very hemorrhagic appearance, had shown very rapid rates of erythrocyte destruction, caused largely by extravasation of blood into the necrotic center of the tumor. Regardless of the pattern of vascular involvement, the erythrocyte destruction and the anemia of the host could be attributed largely to the loss of blood into the area of the tumor.

The question arises as to what extent similar vascular damage can account for the erythrocyte destruction and the anemia frequently observed clinically in patients with malignant tumors. The problems of executing such investigations have been discussed elsewhere (*9*). It should be remarked that a cursory review of large collections of slides of human tumors would not be sufficient to reach even a preliminary solution to the problem, since most pathologic material has been collected for the primary purpose of diagnosing the type of tumor present. To do this, the pathologist naturally selects the white, firm tumor nodules for sectioning, from which he can attain the maximum accuracy in diagnosis. Therefore, in carrying out a study of the vascular lesions of human tumors in relation to the erythrocyte destruction and anemia of the host, it is important to obtain representative samples of all portions of the tumor.

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PLATE 82

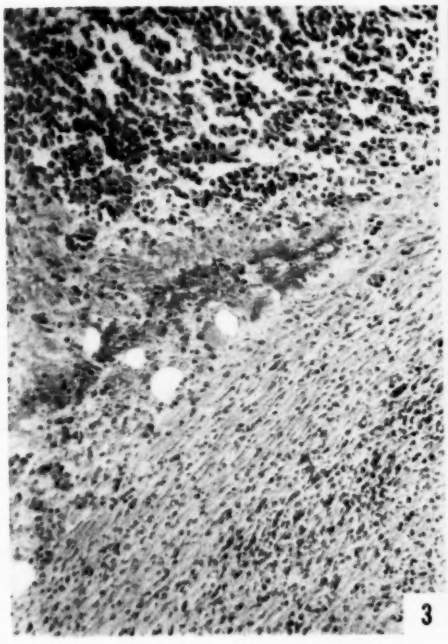
Areas of hemorrhage in tissue sections of Lymphosarcoma R2788, 29 days after subcutaneous implantation into a M520 × (A × C)/9935 male rat. In connection with iron localization studies described in text, host had received intravenous injections of erythrocytes labeled with 0.38 μc of Fe^{59}, 22 days after inoculation of the tumor, and erythrocytes tagged with 1.7 μc of Cr^{51}, 30 minutes before death. These photographs were all taken from this one tumor. Hematoxylin and eosin. 6 μ

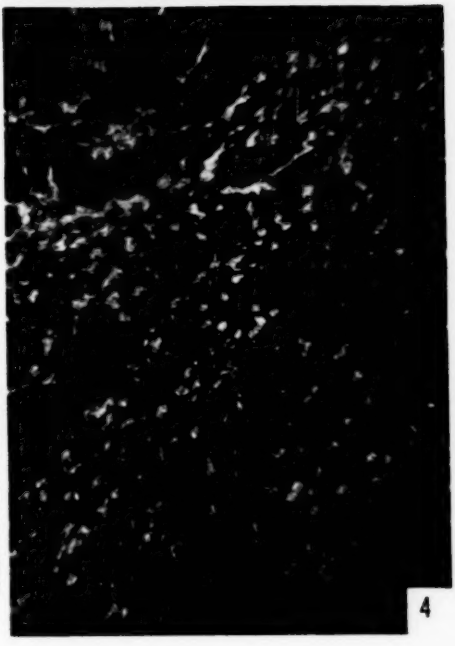
FIGURE 1.—*Note* band of extravasated blood between tumor nodules and beneath the capsule.

FIGURE 2.—Showing macrophages filled with hemosiderin in connective tissue adjacent to hemorrhagic area. This figure is a magnified section taken from the upper left portion of figure 1, and has been rotated 180°.

FIGURE 3.—Section showing tumor tissue, a triangular hemorrhagic area, and a connective-tissue trabecula with macrophages filled with hemosiderin.

FIGURE 4.—Microincinerated section immediately adjacent to section shown in figure 3. *Note* rich deposits of iron (red ash) in connective-tissue trabecula.





Price *et al.*

The Histopathology of the Macroglobulinemia of Waldenström[1]

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Summary

The histopathologic, phase microscopic, and clinical pathologic features of macroglobulinemia are described. The presence of lymphoid plasma cells containing intranuclear periodic acid-Schiff positive material is noted in two cases with the macroglobulinemia of Waldenström and in one with the Bing-Neel syndrome. It is suggested that this material and the intranuclear "vacuoles" observed in phase microscopy are chemically identical to the circulating 18S, hexose-rich γ-macroglobulin. The classification of the macroglobulinemia of Waldenström as a neoplasm of reticuloendothelial origin is mentioned and the differentiation between this disease, lymphoma, and multiple myeloma is discussed.—**J. Nat. Cancer Inst. 22: 887-917, 1959.**

This paper describes the histopathologic features of 3 cases of proved macroglobulinemia—2 of the macroglobulinemia of Waldenström and 1 of the Bing-Neel syndrome. The presence, in each case, of a particular cell type containing intranuclear and intracytoplasmic periodic acid-Schiff (PAS) positive material is noted.

In 1944 Waldenström (*1*) reported 3 patients whose serum contained an increased amount of serum macroglobulin. Salient clinical features of these 3 patients were fatigue, epistaxis, gingival hemorrhage, and a moderate lymphadenopathy. There was an anemia, a markedly elevated erythrocyte sedimentation rate, a bone marrow lymphocytosis, and a hyperglobulinemia. Waldenström named this syndrome, macroglobulinemia (*2*).

Bichel, Bing, and Harboe (*3*) reported a similar case with macroglobulinemia in 1950. Their patient had visual disturbances, paresthesias, and paresis in addition to fatigue and hemorrhages. Many of the organs and

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[2] Pathologic Anatomy Branch.

[3] Metabolism Service.

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[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

tissues of this patient, including the Virchow-Robin spaces of the cerebral vessels, were infiltrated by peculiar lymphocytoid cells. In 1936 and 1937, Bing and Neel (*4, 5*) noted 3 patients with similar clinical and histologic findings. Although ultracentrifugal analyses of the serum from these 3 patients were not performed, the coincidence of macroglobulinemia and central nervous system affection has been designated the Bing-Neel syndrome (*6, 7*).

Report of Cases

CASE 1[6]

Clinical summary (00–22–58).—This 83-year-old white woman had anemia, hepatosplenomegaly, and a weight loss of 16 pounds in February, 1953. When first seen at the Clinical Center, in October 1954, she was thin, pale, and had hepatosplenomegaly and generalized moderate lymphadenopathy. There had been no significant hemorrhages.

The hemoglobin was 4.9 gm. percent; the white blood cell count, 7,200 per mm.^{3} with 30 percent polymorphonuclears and 70 percent lymphocytes; the platelet count was 16,000 per mm.^{3} The erythrocyte sedimentation rate was not determined. Urinalysis was normal. The total serum protein was 7.8 gm. percent with 2.2 gm. percent albumin and 5.6 gm. percent globulin. Thirty-eight percent of the serum protein (2.9 gm. %) consisted of a macroglobulin ($S^0_{20_w}$ = 18S)[7] which migrated electrophoretically as a γ-globulin. Cryoglobulins were not demonstrable. Dilution of the serum in water (Sia test) produced a heavy white flocculus. The cephalin flocculation was 4 plus and the thymol turbidity was 13 units. Other liver function tests were normal. Skeletal X rays revealed hypertrophic arthritis of the spine and no osteolytic lesions.

Repeated attempts at bone marrow aspiration were unsuccessful. Marrow obtained from an open biopsy of the iliac crest contained large numbers of cells that appeared to be mature lymphocytes. This biopsy was interpreted as consistent with a diagnosis of chronic lymphocytic leukemia.

A clinical and hematologic remission coincident with cortisone administration occurred. This remission lasted for about 1½ years. In the summer of 1956, the patient again developed hepatosplenomegaly, lymphadenopathy, anemia, thrombocytopenia, and lymphocytosis. Terminally, she developed a monilial pharyngitis and died on January 19, 1957, following aspiration of vomitus.

Bone marrow aspiration.—Three weeks before death an adequate specimen of sternal marrow was easily obtained. This marrow was markedly hypercellular and about 85 percent of the cells were lymphoid. In Giemsa-stained smears, many of the cells resembled plasma cells with an eccentric nucleus and an ample, bright-blue cytoplasm which frequently contained a paranuclear clear area (figs. 1 and 2). The nuclear chromatin pattern, however, was diffuse like that of a lymphocyte rather than clumped along the nuclear membrane in a "wheelspoke" fashion like that of a plasma cell. A few of these lymphoid plasma cells contained a rather large, pale-pink area within the nucleus and some had areas within the cytoplasm which stained brilliantly red. With the phase illumination (figs. 3 to 8) the nuclear outline was more irregular than that of a normal

[6] This case will be published in greater detail as a Clinicopathologic Conference in the *Annals of Internal Medicine.*

[7] This designation indicates that the material analyzed in the ultracentrifuge had a sedimentation coefficient of 18S when calculated on a basis of water as the diluent, 20° C. as the temperature, and extrapolated to zero concentration.

plasma cell and the nuclear membrane was thin like that of a lymphocyte. The mitochondria were arranged in a paranuclear crescent as is characteristic of plasma cells. Several cells had round "vacuoles" or irregular "canaliculi" within the cytoplasm (figs. 6 and 7) and an occasional cell seemed to be pinching off some of this intracytoplasmic material (fig. 8). Round "vacuoles" were occasionally noted within the nucleus (fig. 3) and at times these appeared to be discharged through the nuclear membrane into the cytoplasm (fig. 5).

Pathologic Findings

Gross Observations

The body was that of a thin, elderly, white woman. There was generalized lymphadenopathy, cutaneous petechiae, and numerous ecchymoses. The liver weighed 1970 gm. and was finely nodular with a prominent lobular pattern. The spleen weighed 600 gm. and was moderately soft and deep purple with only a suggestion of follicles and trabeculae. The bone marrow was deep red and no lesions were noted.

Microscopic Observations

Bone marrow (*fig. 9*).—The bone marrow was hypercellular. Most of the marrow was occupied by morphologically normal mature and immature lymphocytes, plasma cells, and other cells that were morphologically intermediate between lymphocytes and plasma cells. These lymphoid plasma cells had an eccentric nucleus with a diffuse nuclear chromatin pattern like that of a lymphocyte. Several of these cells contained eosinophilic, PAS-positive material within the nucleus and sometimes within the cytoplasm. Granulocytes, megakaryocytes, and erythroid cells were reduced, mast cells (basophils) were increased, and hemosiderin was in macrophages and extracellular deposits.

Spleen.—The lymphoid follicles were small and composed of morphologically normal immature and mature lymphocytes. The cords of Billroth contained a cellular infiltrate similar to that within the bone marrow. Lymphoid plasma cells were scattered singly and in small aggregates throughout the cords of Billroth. Several of these contained intranuclear and intracytoplasmic PAS-positive material. Hemosiderin was within macrophages and extracellularly.

Lymph nodes (*figs. 10 and 11*).—The architectural pattern was altered by an infiltrate of lymphocytes, plasma cells, and lymphoid plasma cells. Many of the latter cells contained intranuclear and intracytoplasmic PAS-positive material. Focal accumulations of intracellular and extracellular hemosiderin outlining the remaining sinuses were frequently seen. The lymph nodes contained a few lymphocytic aggregates but no follicles.

Liver (*fig. 12*).—The portal areas were densely infiltrated by lymphocytes, plasma cells, and lymphoid plasma cells. Lymphoid plasma cells with intranuclear PAS-positive material were especially numerous in

these areas. Hemosiderin deposits were also present in the portal areas. There was no fibrosis.

General.—Infiltration of other organs and tissues was not found. An aspiration pneumonitis was present. Incidental findings included a terminal bronchial adenoma, carcinoid type; a carcinoid tumor of the stomach, a hamartoma of the kidney, and multiple cavernous hemangiomas of the small bowel and liver. The plasma cells and lymphocytes that were in the tissues of the gastrointestinal tract appeared normal. Globules of extracellular PAS-positive material were occasionally seen in the tissues infiltrated by lymphoid plasma cells, and the intravascular plasma and the pulmonary edema fluid were strongly PAS-positive.

CASE 2

Clinical summary (01–87–64).—This 43-year-old white man was referred to the Clinical Center, by Dr. Edward Adelson, Washington, D.C., and Dr. Norman Shoemaker, Silver Spring, Maryland, upon confirmation of their diagnosis of macroglobulinemia.

The patient was apparently well until early 1957 when a profuse gingival hemorrhage occurred after routine dental prophylaxis. A short time later bleeding ulcers of the oral and pharyngeal mucosae appeared.

In October, 1957, the significant physical findings were a marked generalized lymphadenopathy, hepatosplenomegaly, oral ulcers, retinal hemorrhages, and tortuous retinal veins.

The hemoglobin was 8.2 gm. percent; the white blood cell count, 6,800 per mm.^{3} with 60 percent polymorphonuclears, 36 percent lymphocytes, and 2 percent monocytes; the platelet count was 210,000 per mm.^{3} The erythrocyte sedimentation rate was 2 mm. per hour. Urinalysis was normal. The total serum protein was 12.9 gm. percent with 2.2 gm. percent albumin and 10.7 gm. percent globulin. Most of the increase in serum protein was due to macroglobulins ($S^0_{20_w}$=18S which migrated electrophoretically as γ-globulin. Cryoglobulins were not demonstrable. Dilution of the serum in water (Sia test) produced no flocculus. Liver function tests were normal. Skeletal X-ray films revealed no osteolytic lesions.

At the present time, 14 months later, the patient has improved symptomatically and returned to work.

Pathologic Findings

An adequate sample of bone marrow was aspirated from the sternum in October, 1957. The number of cells appeared to be normal. Approximately 40 percent of the nucleated cells were granulocytes. Megakaryocytes were adequate but erythroid elements were decreased. Mast cells (basophils) were not increased. There were several lymphocytes, a few plasma cells and about 20 percent of the cells were morphologically intermediate between lymphocytes and plasma cells. PAS-positive material could not be demonstrated in these cells. Hemosiderin was not present.

Axillary lymph nodes were excised in November, 1957. These were brownish-gray, firm, and measured approximately 1 × 0.5 × 0.5 cm. The normal architecture was almost completely absent. Aggregates of normal mature lymphocytes were in the cortical area but there were no definite lymphoid follicles. Many lymphoid plasma cells, several of which contained intranuclear and intracytoplasmic PAS-positive material,

were in the sinusoids and around vessels. Globules of extracellular PAS-positive material were occasionally seen in the sinusoids. Hemosiderin deposits were frequent. Mast cells were rare. The intravascular plasma was strongly PAS-positive.

CASE 3

Clinical summary (01-73-76).—This 60-year-old white woman was admitted to the Clinical Center in May, 1957, with a 1-year history of cloudy vision. Several months following the onset of the visual disturbance, a right chorioretinitis was noted. A tuberculin skin test was positive. She had received antituberculosis therapy, and prednisone and ACTH without improvement of vision.

On admission she had cloudy vision, vertex headaches, periodic weakness, and mental depression. The optic fundi were normal except for chorioretinitic lesions in the inferior temporal quadrants. The anterior chambers contained a mild cellular infiltrate and both vitreous bodies contained extensive cellular infiltrates and weblike floaters. Hepatosplenomegaly was noted; lymphadenopathy was not present. There had been no significant hemorrhages.

The hemoglobin was 12.5 gm. percent; the white blood cell count, 5,800 per mm.^{3} with 37 percent polymorphonuclears and 55 percent lymphocytes; the platelet count was 172,000 per mm.^{3} The erythrocyte sedimentation rate was 89 mm. per hour. Urinalysis was normal. The total serum protein was 6.4 gm. percent with 2.9 gm. percent albumin and 3.5 gm. percent globulin. Serum electrophoresis revealed increased γ-globulins, and on ultracentrifugation there was a macroglobulin ($S_{20_w}^0$=18S) which comprised 15 percent of the total serum protein. Cryoglobulins were not demonstrable. Dilution of the serum in distilled water (Sia test) produced a heavy white flocculus. Liver function tests were normal. X rays of the skeleton revealed osteoarthritis in multiple sites.

Constant severe headaches, disorientation, aphasia, and weakness of the right arm developed. An electroencephalogram was compatible with an expanding lesion in the left temporoparietal area. The intracranial pressure was markedly increased and despite an attempt at decompression the patient became comatose and expired.

Pathologic Findings

Gross Observations

The body was that of an obese white woman. There were a few small ecchymoses at venipuncture sites. The liver weighed 2600 gm. and the lobular pattern was accentuated. The spleen weighed 720 gm. and contained prominent lymphoid follicles that measured 1 to 2 mm. in diameter. The mesenteric and periportal lymph nodes were moderately enlarged, gray-white, and firm. Other lymph nodes were not remarkable. The bone marrow was deep red and no lesions were noted.

The brain weighed 1400 gm. after fixation. The left occipital pole was enlarged and extended 4 cm. posterior to the right occipital pole. There was uncal herniation with widening and flattening of the inferior temporal gyri on the left. A soft, gray-white tumor in the left cerebral hemisphere extended from the level of the optic chiasma to within 1 cm. of the occipital pole. In the region of the hippocampus and tail of the caudate nucleus the tumor was necrotic and filled the inferior and posterior horns of the left lateral ventricle (fig. 16). It had eroded through the cortex of the left inferior temporal lobe and compressed the brain stem

at the level of the pons. A globular mass 1 cm. in diameter was found in the right frontal cortex. The other organs and tissues were not remarkable.

Microscopic Observations

Bone marrow (fig. 13).—The over-all cellularity was at the upper limit of normal. Granulocytes were slightly reduced in number and mast cells (basophils) were infrequently seen. Megakaryocytes and erythroid elements appeared to be normal. There was a small amount of hemosiderin. The most striking feature of the marrow was the presence of numerous morphologically normal lymphoid follicles with germinal centers. The reticulum cells within the germinal centers appeared to be normal. There were scattered focal aggregates of lymphocytes, plasma cells, and lymphoid plasma cells. These latter cells frequently contained intranuclear and intracytoplasmic PAS-positive material.

Spleen.—The splenic architecture was accentuated by an increased number of large lymphoid follicles and enlargement of the cords of Billroth. Central arterioles were not seen in all of the lymphoid follicles. Some of the follicles contained germinal centers with morphologically normal reticulum cells. The enlargement of the cords of Billroth was due to the presence of mature and immature lymphocytes (60%), plasma cells (20%), and lymphoid plasma cells (15%). Several of the lymphoid plasma cells contained intranuclear and intracytoplasmic PAS-positive material.

A few large cells, considered to be abnormal reticulum cells, were interspersed, as single cells or small aggregates, in the cords of Billroth and in the peripheral two thirds of the lymphoid follicles (fig. 14). They had a slightly irregular to deeply cleft, hyperchromatic nucleus with a diffuse, nuclear chromatin pattern and prominent, irregular, eosinophilic to amphophilic nucleoli. The cytoplasm was amphophilic, irregular, and moderate to abundant in amount. Mitoses and binucleate forms were frequently seen in these reticulum cells. Mast cells were rarely seen and hemosiderin deposition was not prominent.

Lymph nodes.—The architectural pattern of the lymph nodes of the mesentery and periportal area was altered by an infiltrate like that in the splenic cords. Lymph nodes from other areas were not remarkable except for variable degrees of reticulum-cell hyperplasia.

Liver.—The portal triads contained numerous small lymphocytes and occasional abnormal reticulum cells, but lymphoid plasma cells were extremely rare. The sinusoids contained a few of the abnormal reticulum cells and several lymphoid plasma cells, but PAS-positive material was not often found in these.

Kidneys (*fig. 15*).—There were cellular infiltrates in perivascular areas within the renal parenchyma and throughout the fat of the renal pelvis. The infiltrate in these locations showed considerable variation in cell size and marked irregularities of the nuclear outline of the smaller lymphocytes. Lymphoid plasma cells, with PAS-positive intranuclear and intracyto-

plasmic material, were frequently seen and tissue mast cells were increased in the areas of the infiltrate.

Central nervous system (*fig. 17*).—The meninges and nearly all the perforating meningeal vessels of the cerebral hemispheres, cerebellar hemispheres, midbrain, pons, and medulla oblongata were involved by an infiltrate which involved the vessel walls and filled the Virchow-Robin spaces. The majority of the cells in these areas were mature lymphocytes but a few mature plasma cells, abnormal reticulum cells (like those in the spleen), and occasional lymphoid plasma cells, with or without the PAS-positive material, were seen.

Vast numbers of the abnormal reticulum cells extended from the external membrane of the Virchow-Robin spaces and infiltrated the brain substance. Frequently, aggregates of these from one focus coalesced with those from neighboring foci. The 2 tumors noted grossly appeared to have been formed in this manner since the abnormal reticulum cells were most concentrated in perivascular locations. The brain substance infiltrated by these cells was necrotic but there was no reactive gliosis. Lymphocytes, plasma cells, and lymphoid plasma cells were extremely rare within the tumor areas and no intracellular PAS-positive material was seen within the abnormal reticulum cells.

General.—Globules of extracellular PAS-positive material were occasionally seen throughout areas infiltrated by lymphoid plasma cells and the intravascular plasma throughout the tissues was markedly PAS-positive.

Discussion

Clinical Findings

Waldenström has recently reviewed some 100 cases of macroglobulinemia which have been published since his original report (*8*). Most of these have appeared in recent years and nearly every month new cases are added to the literature. Thus, the clinical findings are becoming relatively well known.

A tabulation of the clinical and clinical laboratory findings in 60 cases (*1*, *3*, *6*, *7*, *9–42*) is presented in tables 1 and 2. In each of these cases the presence of increased macroglobulins was confirmed by ultracentrifugal studies. Two thirds of the cases reported were in men and two thirds of all the patients were between 50 and 70 years of age at the time of diagnosis.

Clinical Laboratory Findings

Many clinical laboratory abnormalities are found in the presence of the increased serum macroglobulins. The erythrocyte sedimentation rate is almost always elevated, partly because of the anemia but also probably due to the presence of large globulin molecules. Rouleaux formation is frequently seen on peripheral blood smears, and the agglutination of cells in the counting chamber often makes a red blood cell count impossible.

TABLE 1.—*Symptoms and signs in 60 cases of the macroglobulinemia of Waldenström*

	Definitely present	Definitely absent	Not stated
Enlarged liver, spleen, or lymph nodes	52	4	4
Fatigue	39	—	21
Mucosal bleeding or purpura	39	15	6
Retinal hemorrhages and dilated veins	23	3	34
Bone pain	3	9	48

The Sia test is a nonspecific test for euglobulins. This test was once thought to be specific for kala-azar but is now known to be positive in some cases of cirrhosis or carcinoma of the liver, tuberculosis, chronic malaria, multiple myeloma, and disseminated lupus erythematosus (*6*, *24*, *43*). The test is carried out by dropping 1 or 2 drops of serum into a cylinder containing distilled water. The immediate development of a white flocculus that rapidly settles to the bottom of the cylinder constitutes a positive test (*43*, *44*). A positive Sia test may be noted in routine clinical chemistry procedures, such as the uric acid and bilirubin analyses, wherein serum is diluted with distilled water. The test was positive in 2 of the 3 cases reported here and, as noted in table 2, has been definitely negative in only 4 of 32 cases.

TABLE 2.—*Clinical laboratory data in 60 cases of the macroglobulinemia of Waldenström*

	Definitely present	Definitely absent	Not stated
Anemia	53	—	7
Leucopenia (less than 5,000 WBC/mm.^{3})	26	25	9
Relative or absolute lymphocytosis—peripheral blood	33	17	10
Thrombocytopenia	21	22	17
Bone marrow interpreted as "abnormal"	56	2	2
Elevated erythrocyte sedimentation rate	50	3	7
Bleeding or coagulation time prolonged	8	20	32
Bence Jones proteinuria	9	27	24
Cryoglobulins	9	21	30
Positive Sia test	28	4	28

The liver flocculation tests are frequently abnormal without abnormalities in other liver function tests. This appears to be related to the presence of abnormally increased amounts of protein and is not necessarily correlated with a positive Sia test.

The occasional presence of cryoglobulins (about 15% of cases) may cause jelling of the serum in the refrigerator. Even without demonstrable cryoglobulins, the blood and serum from patients with macroglobulinemia is often viscous.

These various peculiarities which may be noted in the clinical laboratory are hints that a patient may have macroglobulinemia but the definitive test is ultracentrifugation of the serum.

Some Characteristics of Macroglobulins

Upon ultracentrifugal analysis the major portion of the normal serum γ-globulins is in the 6.6S (7S)[8] component, and has a molecular weight of approximately 160,000. Macroglobulins have a sedimentation constant of 15S or greater and a molecular weight of approximately 1 million. Normally, about 4 percent (0.3 gm.%) of the total serum protein is macroglobulin. Ultracentrifugation of the serum from the patients reported here revealed that macroglobulins comprised 38, 55, and 15 percent of the total serum protein. The sedimentation constant for the major macroglobulin component in each case was approximately 18S.

Most of the normal 6.6S γ-globulins have a hexose content of 1 to 2 percent and macroglobulins contain 4 to 5 times this amount of hexose (*45*). Some myeloma proteins have been reported as having a hexose content over 2 percent but this does not approach the hexose content of macroglobulins (*46*). The hexose content of purified serum macroglobulins from the patients reported here was 6.1, 5.7, and 4.5 percent. Macroglobulins and myeloma proteins migrate with the mobility of γ- or β-globulins on electrophoretic examination and cannot be distinguished from each other by this technique. However, the high carbohydrate content of macroglobulins can be demonstrated by paper electrophoresis utilizing modified PAS staining techniques and this is sometimes a useful means of distinguishing macroglobulins from myeloma proteins (*18*, *46*).

Histopathology

The histopathologic findings in most of the reported cases of macroglobulinemia consist of descriptions of aspirated bone marrow or biopsied lymph nodes. In our review of the literature, reports of 13 autopsied cases (*3*, *10*, *12*, *16*, *25*, *26*, *38*, *40*, *42*, *47–49*) were found. Histopathologic alterations were noted within the central nervous system in only one of these (*3*).

Pathologic alterations are always found in some combination of lymph nodes, bone marrow, or spleen but the organ most severely affected differs from case to case. Infiltrates of variable cell types have been described in a wide variety of tissues including the liver, kidney, adrenal gland, lung, striated muscle, pituitary gland, pancreas, and brain. The infiltrate is almost always diffuse but 3 cases of tumor formation or osteolytic bone lesions have been described (*32*, *50*, *51*). The third patient reported here had a large cerebral tumor but no osteolytic bone lesions.

The diagnoses on the basis of examination of tissues prior to ultracentrifugation of the serum have ranged from normal to reticulum-cell sarcoma. The most frequent diagnoses have been: "lymphoma, mature cell type" and "diffuse multiple myeloma," but "reticulum-cell hyperplasia" has been often mentioned, and, in rare instances, Hodgkin's disease has

[8] The observed sedimentation constant for γ-globulin will depend on the conditions of ultracentrifugation analysis. Analyses in our laboratory indicate a sedimentation constant of 6.6S, although the 7S value is often seen in published reports.

been considered. The diagnosis in Case 1 of the patient reported here had been chronic lymphocytic leukemia on the basis of a bone marrow biopsy.

This diversity of diagnoses is due to the variability of the cellular infiltrate. There is usually one predominant cell type within the infiltrate and an associated proliferation of other cells of the reticulum-cell, lymphocyte, or plasma-cell type. The predominant cells may resemble either mature lymphocytes or mature plasma cells and this feature accounts for the frequency of the diagnosis of lymphoma or diffuse multiple myeloma.

Bichel, Bing, and Harboe (*3*) and Tischendorf and Hartmann (*9*) recognized a particular cell type, which has since been noted in many of the reported cases. This cell type has been called a lymphoid plasma cell and it superficially resembles an immature or mature plasma cell. It has an eccentric round to ovoid nucleus and an ample cytoplasm within which there is occasionally a paranuclear clear area. In contrast to the normal plasma cell the nuclear outline is often slightly irregular and the nuclear chromatin is diffusely arranged. Electron microscopic observations by Braunsteiner, Fellinger, and Pakesch (*52*) have revealed a prominent cytoplasmic ergastoplasm in typical lymphoid plasma cells from 2 patients with macroglobulinemia. Since a prominent cytoplasmic ergastoplasm is now regarded as a characteristic of the plasma cell (*53*), this finding, if confirmed in other cases of macroglobulinemia, may be evidence for a closer histologic relationship between macroglobulinemia and multiple myeloma than between macroglobulinemia and the lymphomas.

On the basis of the predominant proliferative cell type, Rohr (*54*) recognizes a lymphocytic type, a plasmacytic type, and lymphoid plasmacytic type of macroglobulinemia.

In 1950, Tischendorf and Hartmann (*9*) and Bichel, Bing, and Harboe (*3*) described an increased number of mast cells associated with the infiltrate and this has since been noted in about 15 percent of the reported cases. Proliferation of mast cells was present in the bone marrow of the patient in Case 1 and in the areas of infiltration in the renal pelvis of the patient in Case 3.

Of particular interest in the 3 cases of Waldenström's macroglobulinemia reported here was the PAS-positive material within the nucleus and cytoplasm of many of the lymphoid plasma cells (figs. 18 to 25). The variable amount of the intracellular material suggests a progressive increase of this material within the cells. In what we consider to be the earliest stages in the production or accumulation of the intranuclear material, the nucleus contained 1 to 4 small, centrally located globules which were surrounded by a clear zone (figs. 18 to 22). These globules appeared to condense and enlarge, crowd the nuclear chromatin to the periphery of the nucleus, and ultimately rupture the nuclear membrane allowing the PAS-positive material to protrude into the cytoplasm (figs. 22 to 24). In some of the cells the material filled the cytoplasm and was encircled by nuclear chromatin (fig. 24). The nuclear chromatin appeared to degenerate and the cytoplasmic membrane appeared to dis-

rupt or degenerate leaving the PAS-positive globule lying extracellularly within the tissues (fig. 25). We believe that the intranuclear and intracytoplasmic "vacuoles" seen in the phase preparations represent the PAS-positive material demonstrated in the tissue sections.

Similar observations of intranuclear and intracytoplasmic material which reacted histochemically as protein have been reported in one case of macroglobulinemia by Schamaun (*47*); in another case, Lehlbach (*38*) noted the strong PAS-positive character of this material. With Schamaun we believe that the intranuclear material may be chemically identical to the serum macroglobulin. Histochemically, the material was identifiable as a glycoprotein by virtue of its staining reactions (*55*). It was Millon-positive; PAS-positive; diastase PAS-positive; toluidine blue-not metachromatic; Alcian blue-negative and Sudan black B-negative. The material contained neither deoxyribonucleic acid with the Feulgen technique, nor ribonucleic acid with the methyl-green pyronin stain; with trichrome stains it was variably blue or red. The intravascular plasma and the extracellular globules lying free within the tissues were histochemically identical to the intranuclear material. As noted previously, purified macroglobulins are proteins with a hexose content greater than 4 percent and, therefore, may be considered to be glycoproteins. Although this histochemical evidence is consistent with an intranuclear location of macroglobulin, the intranuclear material has not been positively identified as macroglobulin. It is hoped that projected immunochemical studies will be helpful on this point.

Ortega and Mellors (*56*), with a fluorescent antibody technique, have studied the intracellular location of γ-globulins. These were in the cytoplasm of cells within the germinal centers of lymphatic follicles and in the cytoplasm of mature and immature plasma cells of two types—those with and those without Russell bodies. They observed no instance of intranuclear localization of the fluorescein-coupled antibody even though their material included sections of a spleen from a patient with macroglobulinemia. However, their studies probably relate primarily to 6.6S γ-globulin formation and their findings should not be regarded as evidence against the intranuclear location of macroglobulin. The γ-globulin used by these investigators to produce antibodies probably contained little, if any, 18S macroglobulin (*57*, *58*) which has distinctly different antigenic properties from the 6.6S γ-globulins (*59*).

Significant numbers of typical lymphoid plasma cells containing intranuclear PAS-positive material have been found only in cases of the macroglobulinemia of Waldenström. However, more observations are needed before the presence of large numbers of these cells can be considered as specific for macroglobulinemia.

Since normal human serum contains macroglobulins, we have looked for similar cells in lymphoid tissues from patients in whom ultracentrifugal analysis revealed no increased amount of serum macroglobulins. Upon extensive searching, we have found occasional cells in these tissues which do contain intranuclear PAS-positive material. The amount of this ma-

terial varies from punctate areas to areas equivalent to about half the size of the nucleus. In an occasional such cell the PAS-positive material lies in apparent contact with the nuclear membrane, but no cells have been seen in which the nuclear membrane is ruptured as it frequently is in our cases of the macroglobulinemia of Waldenström.

Material from other diseases examined for cells containing PAS-positive intranuclear material has included 15 cases of multiple myeloma and 15 cases of lymphoma. No typical cells were seen in these cases. The lymphomas included acute and chronic lymphocytic leukemia, Hodgkin's disease, reticulum-cell sarcoma, and mycosis fungoides. Also, 1 case of idiopathic cryoglobulinemia without macroglobulinemia, the spleen from a patient with kala-azar, and lymph nodes from 2 patients with acute toxoplasmosis did not have a significant number of typical cells. Rappaport and Johnson (*60*) reviewed 100 cases of malignant lymphoma in a study of intracellular PAS-positive material. They found such material in the cytoplasm of plasma cells and reticulum cells in 14 cases in which there had been an associated hemolytic anemia. Through the courtesy of Drs. Rappaport and Johnson we have been privileged to review this material. In 2 of the 14 cases, in both of which there had been hyperglobulinemia, cryoglobulinemia, and a clinical course compatible with macroglobulinemia, intranuclear PAS-positive globules were found in several lymphoid plasma cells.

Differential Diagnosis

The certain diagnosis of macroglobulinemia rests upon the demonstration of increased serum macroglobulins by ultracentrifugal analysis. Histologically, the differential diagnosis is usually between lymphoma, myeloma, and macroglobulinemia. The pleomorphism of the cellular infiltrate is usually sufficient to cause hesitancy in making the diagnosis of a mature cell lymphoma, even in instances of almost complete loss of the normal lymph node or bone marrow architecture as in Case 1 of this series. In instances of partial alteration of the normal architecture, the remnants of lymphoid follicles contain morphologically normal lymphocytes whereas the sinuses contain the pleomorphic infiltrate. This sparing of lymphoid follicles was particularly apparent in Case 3.

The histologic features which differentiate macroglobulinemia from multiple myeloma are the pleomorphism of the infiltrate and the diffuse pattern of the infiltrate without the formation of frank tumors, except in rare instances. Bence Jones proteinuria has been found in several cases of macroglobulinemia, but the renal alterations frequently found in multiple myeloma have not been mentioned.

The Bing-Neel syndrome appears to result from involvement of the central nervous system by the infiltrate of the macroglobulinemia of Waldenström. Cerebral tumors composed of abnormal reticulum cells have not been previously described in association with macroglobulinemia, but massive perivascular accumulations of lymphocytes and reticuloendothelial cells were noted in the central nervous system tissues of the patients

reported by Bing and coauthors. The description of the other tissues from Bing's cases differs from our observations in only a few minor aspects.

The cerebral tumors in our case are consistent with a diagnosis of reticulum-cell sarcoma of the brain (microgliomatosis) and it would be of interest to know whether other cases of this disease had an associated macroglobulinemia.

Classification of Macroglobulinemia

On the basis of the histologic alterations of the tissues affected by the pleomorphic infiltrate as well as consideration of the uniformly fatal outcome of the disease, we believe that macroglobulinemia is a neoplastic proliferation of reticuloendothelial origin. This neoplasm has been considered to be either a variant of multiple myeloma or a type of lymphoma.

A satisfactory classification of macroglobulinemia is complicated by the differing clinical aspects, by the variability of the histologic findings, and by the differing physicochemical properties of the macroglobulins, as reported in the published cases of the macroglobulinemia of Waldenström.

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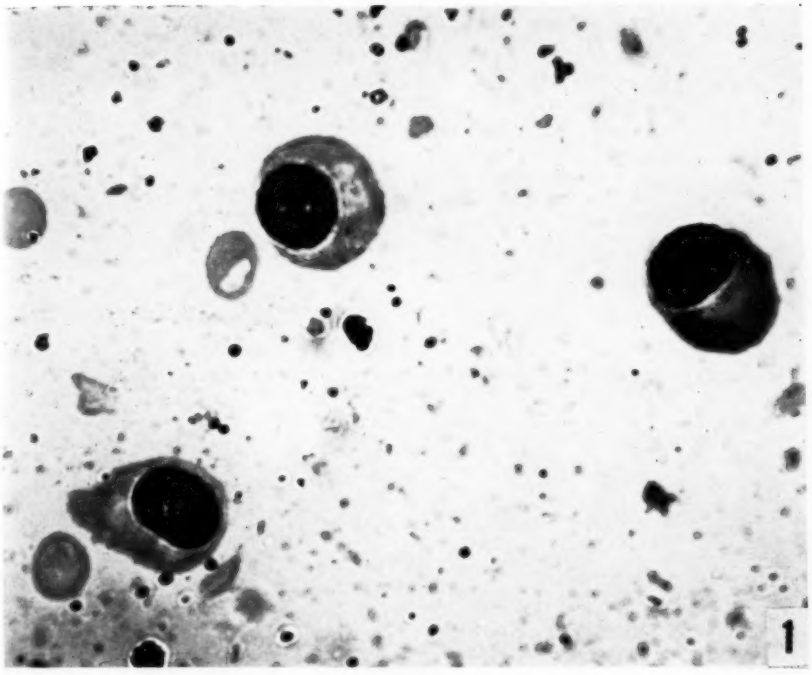
PLATE 83

FIGURE 1.—Case 1. Bone marrow smear. Three typical lymphoid plasma cells with an eccentric nucleus, diffuse nuclear chromatin pattern, and a large cytoplasm with a paranuclear clear area. Two hemosiderin granules are visible. Giemsa stain. × 1,280

FIGURE 2.—Case 1. Bone marrow smear. Two typical lymphoid plasma cells, 1 lymphocyte, 1 mast cell (basophil), and 1 lymphoid nucleus. *Compare* lymphocytic nucleus with those of the lymphoid plasma cells. Giemsa stain. × 1,280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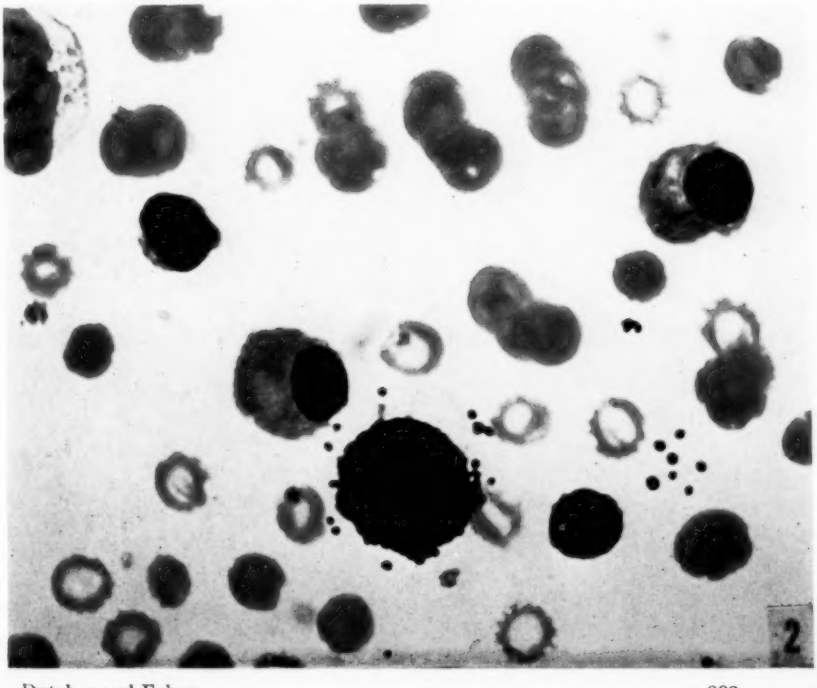
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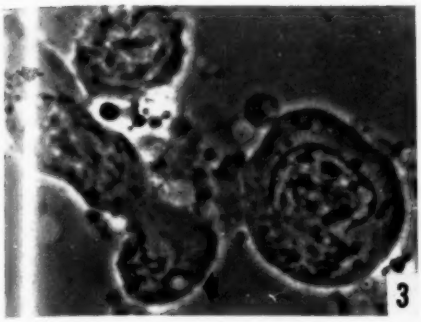
PLATE 84

FIGURES 3 to 8.—Case 1. Bone marrow aspirate. Phase illumination. Figure 4 is a photomicrograph of the same field as figure 3 taken several minutes later. The intranuclear and intracytoplasmic "vacuoles" in the elongated cell of figure 3 are absent in figure 4. Figure 5 shows an irregular cell, which contains some intranuclear material, and another "vacuole" appears to be partially within the nucleus. Figures 6 and 7 show cells with small and large cytoplasmic canaliculi. Cell in figure 8 appears to be "pinching off" an intracytoplasmic globule.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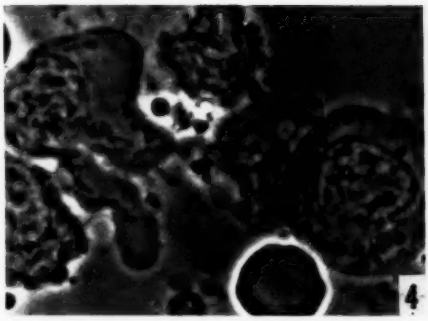
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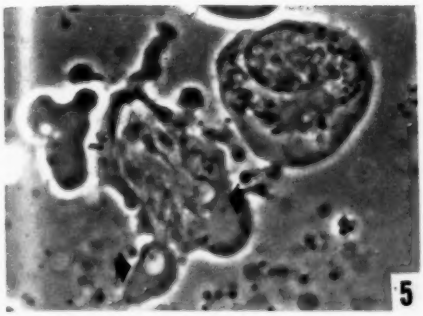
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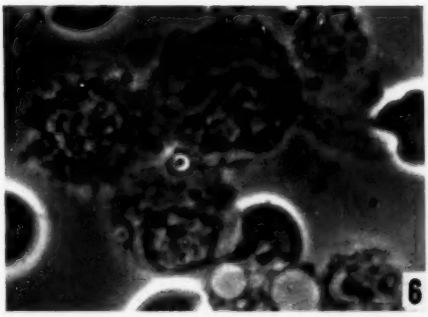
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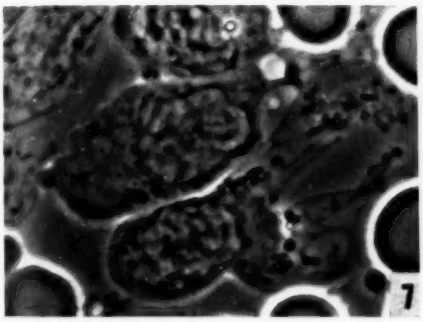
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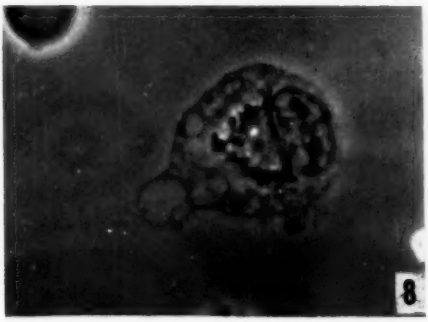
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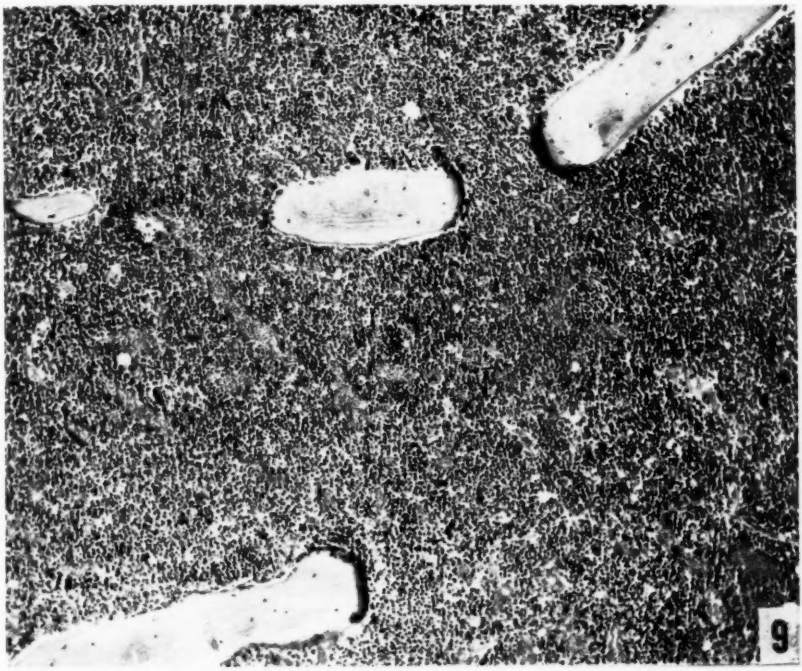
PLATE 85

FIGURE 9.—Case 1. Bone marrow section. Autopsy material. Marrow is markedly hypercellular. A few islands of erythropoiesis and an occasional megakaryocyte are recognizable. Hematoxylin and eosin stain. × 95

FIGURE 10.—Case 1. Lymph-node section. Architectural pattern is altered by a cellular infiltrate which extends into the perinodal fat in the *upper right* corner. *Scattered black areas* are hemosiderin deposits. Hematoxylin and eosin stain. × 75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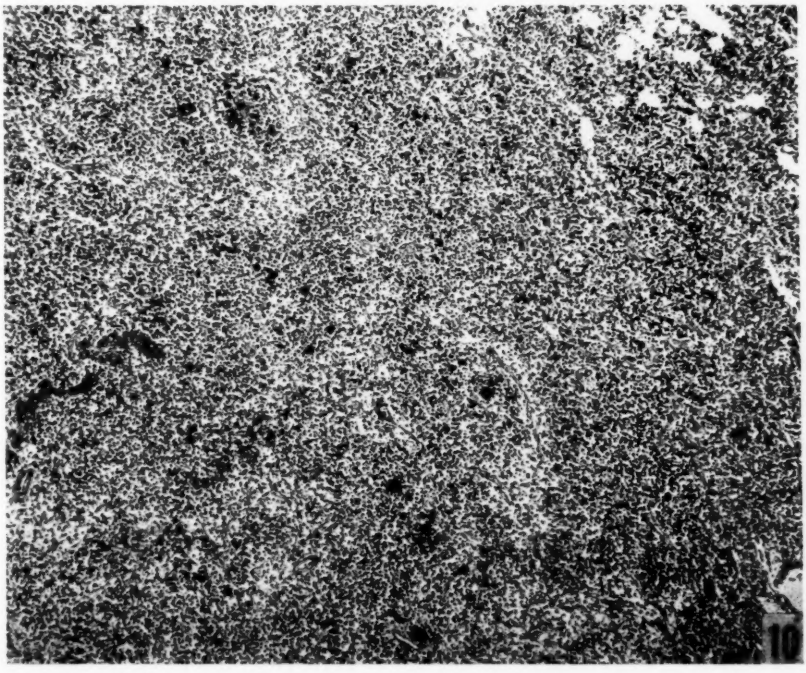
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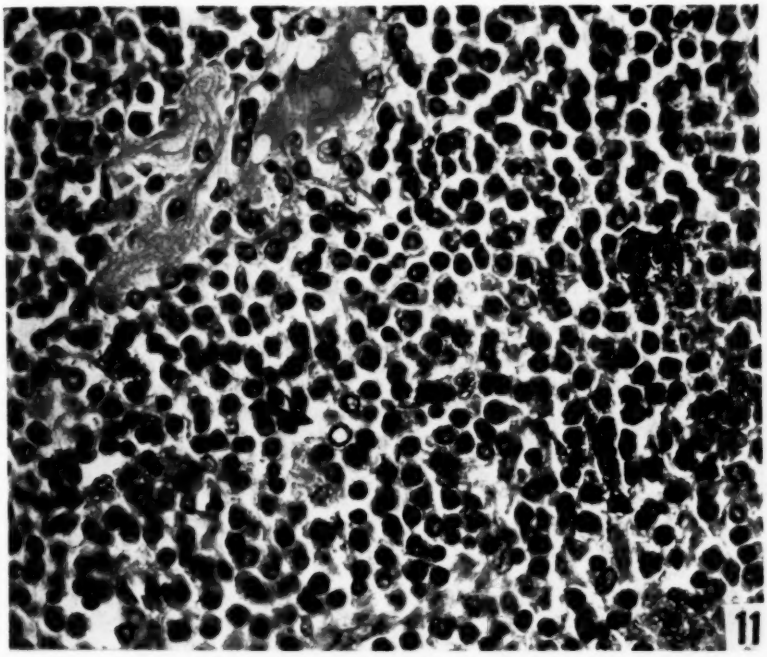
PLATE 86

FIGURE 11.—Case 1. Lymph-node section. Infiltrative cells consist of lymphocytes, plasma cells, and lymphoid plasma cells. *Note* intranuclear material in cell near *center*. Numerous cells contain hemosiderin. Hematoxylin and eosin stain. × 520

FIGURE 12.—Case 1. Liver section. Portal area is heavily infiltrated by lymphocytes, plasma cells, and lymphoid plasma cells. Intranuclear material is present in 2 cells. Several deposits of hemosiderin are present. Hematoxylin and eosin stain. × 440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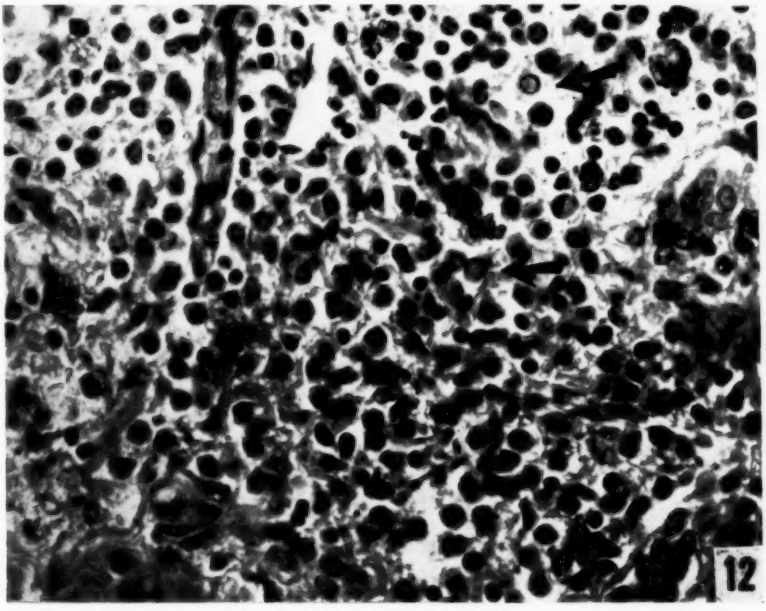
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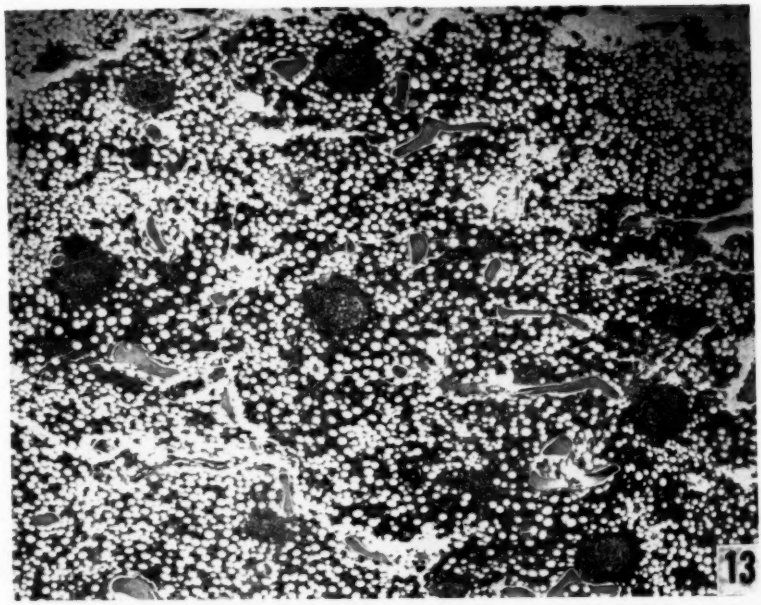
PLATE 87

FIGURE 13.—Case 3. Bone marrow section. There are 7 lymph follicles in this field. Each follicle is composed of apparently normal lymphocytes and has a germinal center of apparently normal reticulum cells. Hematoxylin and eosin stain. × 13

FIGURE 14.—Case 3. Spleen section. Splenic sinuses and cords of Billroth contain many plasma cells and lymphoid plasma cells. A few, large, abnormal reticulum cells are present. Hematoxylin and eosin stain. × 510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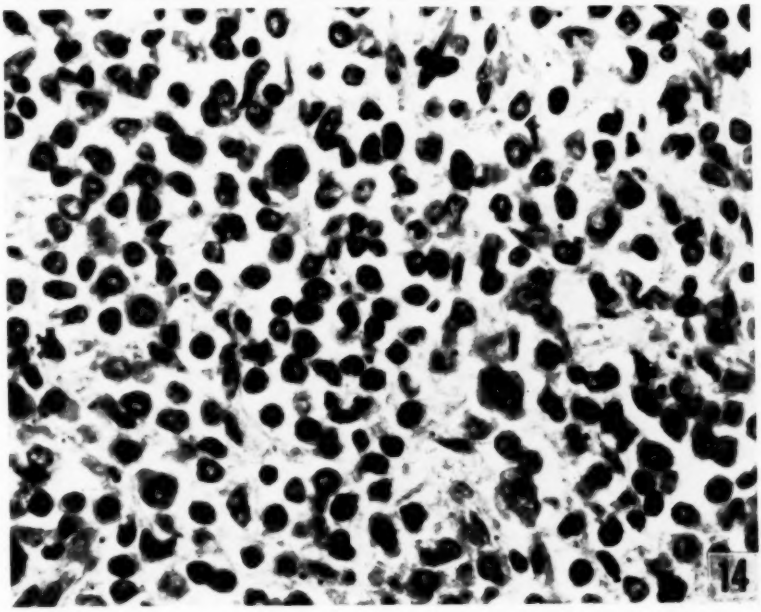
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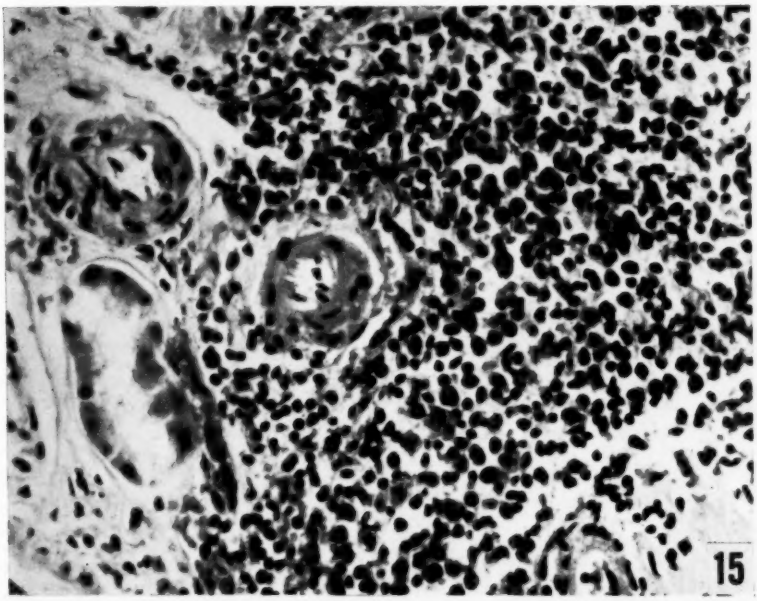
PLATE 88

FIGURE 15.—Case 3. Kidney section. Infiltrate is predominantly perivascular and contains a variety of cell types. There are abnormal reticulum cells, lymphocytes, plasma cells, lymphoid plasma cells, and mast cells. Hematoxylin and eosin stain. × 267

FIGURE 16.—Case 3. Transverse section of brain. Left cerebral hemisphere is involved by a large tumor which has obliterated the ventricle at this level. There is a marked left uncal herniation with compression of the medulla oblongata. Areas of hemorrhage above tumor are secondary to an aspiration biopsy.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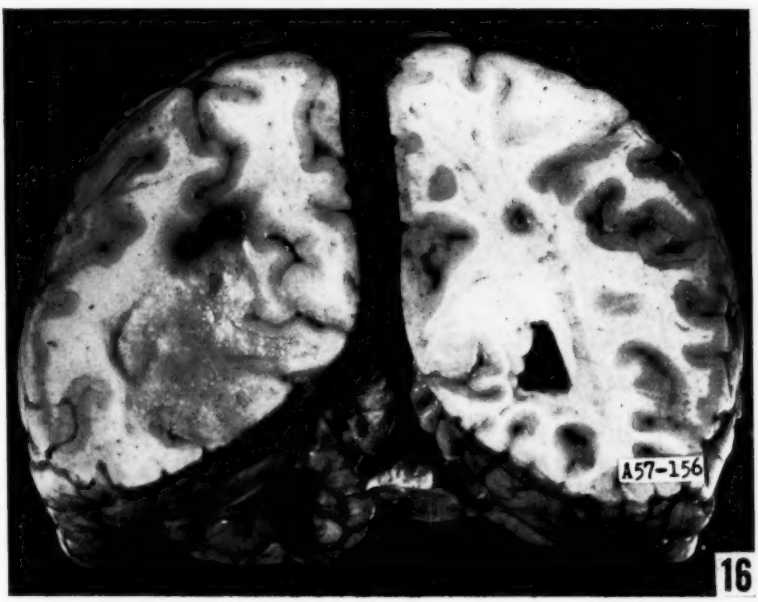
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PLATE 89

FIGURE 17.—Case 3. Left temporal cortex of brain. There is a dense cellular infiltrate of the meninges and Virchow-Robin spaces composed of lymphocytes, plasma cells, lymphoid plasma cells, and an occasional abnormal reticulum cell. There are perivascular accumulations of abnormal reticulum cells and the tumor (*at left*) consists almost exclusively of abnormal reticulum cells. Hematoxylin and eosin. × 50

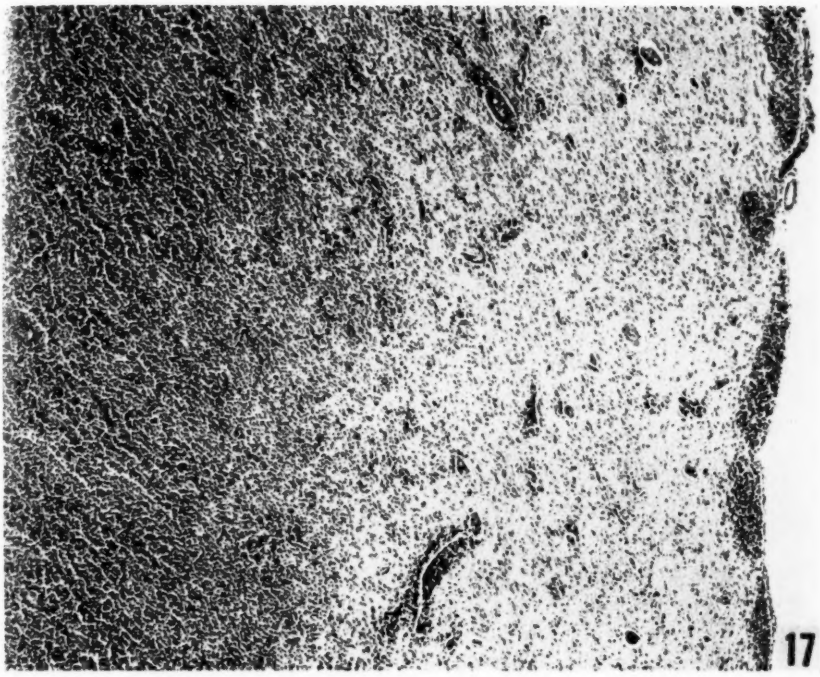
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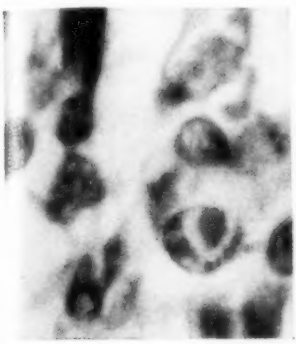
PLATE 90

FIGURES 18 to 25.—Cases 1, 2, and 3. These photomicrographs show the postulated increase in the intranuclear PAS-positive material with eventual rupture through the nuclear membrane into the cytoplasm and finally into the tissues as extracellular PAS-positive globules. Red streak at *upper left* of figure 18 is a blood vessel (out of focus) containing PAS-positive plasma. The red-orange material at *top* of figure 19 is hemosiderin. Periodic acid-Schiff stain. × 2,000



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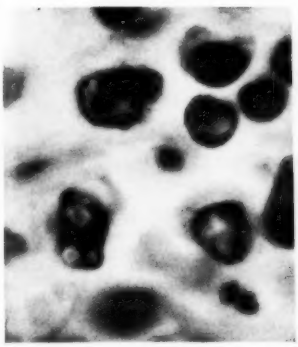

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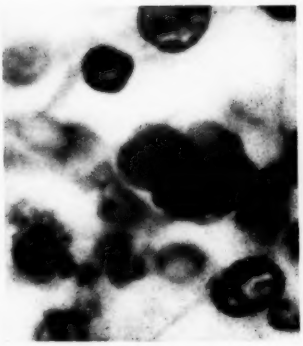

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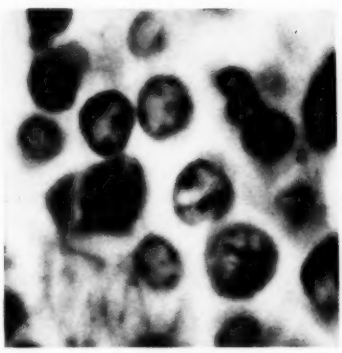

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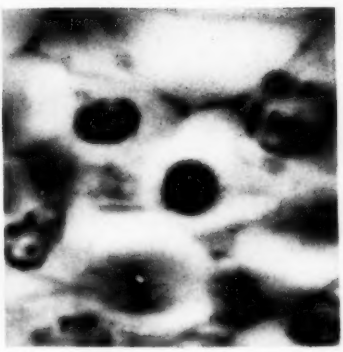

25

Detection of Antigenically Distinctive Components of Mouse Melanoma S91[1,2]

PETER ABRAMOFF, HARRY CHINCHINIAN,[3] *and* JOHN W. SAUNDERS, JR.,[4] *Department of Biology, Marquette University, Milwaukee, Wisconsin*

Summary

Antiserums were produced in White Rock and New Hampshire Red cockerels against the S91 mouse melanoma and against tissues of normal mice of the inbred tumor-host strain (DBA/1). After absorption with normal mouse-tissue antigens, antiserums against the neoplasm showed significant precipitation titers of specific antibodies in reaction with the tumor antigen. Likewise, antiserums against normal mouse tissues precipitated normal tissue antigens after absorption with tumor antigen. It is clear, therefore, that the S91 melanoma and normal tissues of the host mouse strain possess distinctive antigenic components. In preliminary tests of a tumor of more recent origin, the B16 melanoma, similar antigenic differences between the neoplasm and tissues of its C57BL/6 host strain were observed.—**J. Nat. Cancer Inst. 22: 919–925, 1959.**

Specific antibodies to particular neoplastic tissues have been demonstrated by several investigators [(*1–6*); *cf.*, also, review by Hauschka (*7*)]. Others, however, have been unable to find evidence of distinctive tumor antigenicity (*8–10*). Since the development of specific anti-tumor serums has significant implications for the selective control of tumor growth, it is of particular importance that the question of neoplastic antigenicity should receive further attention.

In attempts to define the immunological specificities of tumors, the common fowl has not been used to any extent as an antibody producer (*10*); this despite the fact that the fowl usually reacts rapidly to single small injections of a variety of antigenic substances, producing antibodies of relatively high titer which reach a peak in 7 to 9 days (*11*, *12*). It would seem highly desirable, therefore, that the immune responses

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[4] The authors gratefully acknowledge the technical assistance of Miss Mary T. Gasseling, Mrs. Mary Jurkewicz, and Mr. Joseph Balsano.

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of the fowl be employed in examining the antigenic properties of tumors as contrasted with those of normal tissues.

To this end, cockerels were injected with preparations of the S91 mouse melanoma; others were similarly injected with antigen preparations of normal mouse tissue. Antiserums against the neoplasm were then shown, by means of reciprocal absorption procedures, to contain some antibodies which react only with tumor antigens. The neoplasm is thus antigenically distinctive. Likewise, normal tissues, although having antibodies in common with the tumor, possess antigenic components which the tumor does not share. These findings are the chief topic of the report which follows.

Materials and Methods

Tumors.—The transplantable S91 mouse melanoma (Cloudman), which originated at the Roscoe B. Jackson Memorial Laboratory and is carried in DBA/1 mice, was used in this study. The tumor stock obtained from the Jackson Laboratory was maintained by mouse-to-mouse transplant in the inbred host strain. Routine transplants were performed aseptically in order to prevent contamination of the tumors with bacterial antigens. Tumors were harvested for preparation of the injection antigen 6 to 8 weeks after implantation.

Preparation of tissue for immunization.—Freshly excised tumors were minced with scissors, suspended in cold (5° C.) 1 percent NaCl (20 gm. of tumor in 100 ml. of the salt solution), homogenized for 1 minute at high speed in a chilled Waring blendor, and centrifuged for 30 minutes at 1900 × g. The residue was then ground for 2 minutes in a Potter-Elvehjem homogenizer, resuspended in the original supernatant, and then treated once more in the Waring blendor for 1 minute. The resulting brei was filtered through 6 or 8 thicknesses of cheesecloth and used immediately for injection. This was the standard procedure for preparing the antigen for injection.

Other procedures, however, produced equally effective antigen preparations. Tumor tissue, fresh-frozen or frozen and stored at −20° C., which had been ground in a mortar with 1 percent NaCl solution (20 gm. %, wet weight of tumor), was injected as a whole homogenate or, after centrifugation, the supernatant was employed as antigen. In subsequent sections, no distinction is made with respect to results obtained by any of the methods used in preparation of the antigen.

These procedures were also employed to prepare tissues of nontumorous mice of the inbred host strain for injection. Normal mice were killed each time tumor preparations were made, and tissues of the heart, lungs, spleen, kidney, and liver were excised and treated concurrently with the tumor tissues.

Production of antiserum.—White Rock and New Hampshire Red cockerels, 5 to 6 months of age, were used throughout the experiments. Antiserum production was elicited chiefly by means of intravenous injections (wing vein) of the appropriate antigens in the ratio of 1 ml. per kg.

of body weight. Alternatively, subcutaneous, intramuscular, and intraperitoneal injections, with and without Freund's adjuvant (*13*), were employed. These procedures were all equally effective in evoking the immune response and, accordingly, the simpler intravenous injections were used routinely.

Blood was withdrawn by cardiac puncture from immunized and control birds that were fasted overnight. Bleedings were made at 2-day intervals over a period of 2 weeks after inoculation. Antiserums were separated by centrifugation and stored at −20° C. for subsequent assay of their antibody titers.

Evaluation of antibody production.—Antigens, prepared as described, were centrifuged at 1900 × g for 30 minutes. For determining antibody titers, 0.5 ml. of the clear supernatant was serially diluted with 0.5 ml. of 14 percent NaCl, which gave a final salt concentration of approximately 8 percent [Goodman *et al.* (*14*) have shown this concentration to be optimal in the chicken antibody system]. To each dilution of the antigen, 0.1 ml. of antiserum was added. The mixtures were shaken, incubated for 1 hour at 37° C. in a water bath, and then placed in the refrigerator at 5° C. overnight. The antibody titer was defined as the last dilution in which a definitive precipitate could be seen. Absorption procedures are described, as appropriate, in the section which follows.

Observations

The initial experiments in this series clearly showed that the chicken responded immunologically to injections of normal and neoplastic mouse-tissue preparations in much the same manner as it did to the injection of purified soluble-protein antigens. A single injection of antigen evoked a peak of antibody production in 7 to 9 days; a second injection of homologous antigen, given several weeks later, elicited a peak antibody production at 5 days which may be maintained for as long as 3 weeks (*cf. 11, 15*).

Pooled samples of normal chicken serum, tested with normal and neoplastic mouse-tissue antigen, gave no detectable precipitin reactions. On the other hand, both the normal and neoplastic tissues elicited appreciable, but low, precipitin titers in all birds (26 New Hampshire Reds; 16 White Rocks) tested. These titers ranged from 1:8 to 1:32 at the peak of antibody production, regardless of the antigen used or of the breed of fowl; most antiserums showed titers of 1:16. Appropriate cross reactions and absorption tests on these antiserums and their homologous and heterologous antigens gave clear evidence of distinctive antigenic components in both neoplastic and normal mouse tissues. This evidence was obtained in tests with both pooled and individual antiserums. Details of a representative test are described in the paragraphs which follow.

Antigen suspensions (supernatants, see previous description) of normal and neoplastic mouse tissues were each mixed with half their volume of 8 percent NaCl, incubated for 1 hour at 37° C., and stored overnight at 5° C. This procedure brought about the nonspecific precipitation of a quantity of melanin (presumably granules of melano-protein) from the

tumor antigen, but produced no precipitate in preparations of normal mouse tissues.

Four ml. of pooled antiserum against the S91 tumor was absorbed with 2 ml. of the normal mouse antigen, treated as described, and then 2 ml. of 14 percent NaCl was added to give a final concentration of approximately 8 percent in the reaction mixture. The latter was incubated and refrigerated overnight as before, producing a white precipitate that was removed by centrifugation. Addition to the supernatant of 0.25 ml. of the antigen elicited no further precipitation, thus indicating that in the previous step antibodies against normal mouse-tissue components of the tumor were completely absorbed.

To determine whether the absorbed antiserum still contained antibodies capable of reacting with antigenic components of the melanoma, 0.25 ml. of the antiserum was then mixed with 0.5 ml. of 11.5 percent NaCl and 0.25 ml. of tumor antigen previously treated with 8 percent NaCl as described. After incubation, refrigeration, and centrifugation, a white residue was found, presumably the precipitation of antigenically distinctive tumor components by tumor-specific antibodies.

The reaction of these antibodies with heterologous neoplastic antigens was next examined. Antigenic suspensions of the mouse tumors, S180 (carried in strain C albino mice) and B16 (melanoma; carried in C57BL/6 mice) prepared in 1 percent NaCl, were treated with absorbed antiserum as described. No detectable precipitation occurred with either antigen, indicating that distinctive components of the S91 melanoma are not present in the other tumors.

Finally, tests were undertaken to determine whether normal mouse tissues possess antigenic components distinctive from those of the S91 tumor. Pooled antiserums against normal mouse tissues were completely absorbed with melanoma antigen, according to the usual procedures. The resulting supernatant, now treated with normal mouse antigen, gave a heavy precipitate. Presumably, therefore, normal mouse tissues possess antigens not shared by the melanoma.

Discussion

These studies demonstrate that the S91 melanoma has components which are immunochemically distinct from those of normal tissues of the inbred host-mouse strain. Likewise, normal tissues are distinctive from the neoplasm but have, in addition, components which are common to both. In view of the high concentration of melano-protein in the S91 tumor, it has been of particular interest to note that the antigenic specificity of the neoplasm is probably not associated with the melanin granule. Indeed, melanin is precipitated from the tumor antigen nonspecifically on simple addition of 8 percent NaCl, and the resulting supernatant produces a creamy white precipitate when treated with either homologous or heterologous antiserum.

In some respects it may be considered not surprising that a tumor such as the S91 melanoma would show antigenic components different

from those of normal mouse tissues. This tumor has been carried by mouse-to-mouse transplant in the DBA/1 strain for over 20 years, and in this time could possibly accumulate antigenic characteristics distinct from those of its host. There is also the possibility of subline differentiation of the host because of mutation within the inbred DBA strain. These considerations raise the question whether tumors of more recent origin likewise have tumor-specific antigens and, indeed, whether the origin of a neoplasm may in any way be correlated with the formation of specific antigenic substances.

With this question in mind, preliminary studies of the B16 melanoma, which originated in the C57BL/6 strain and was first transplanted January 27, 1955, were undertaken. Antiserums against this tumor, prepared as described in the foregoing sections of this report and absorbed with antigens prepared from normal C57BL/6 mice, showed flocculation titers of 1:16 to 1:32 in reaction with their homologous antigen. These tests were made in January, 1958. Clearly, this melanoma of recent origin may be, at present, as distinctive antigenically from its host strain as the S91 melanoma is, which was first transplanted in 1937.

The question may be raised whether, in these results, we are dealing with organ-specific antigens rather than tumor-specific antigens, for the normal tissues used for absorption and control purposes are not necessarily those from which the tumor arose and thus might be expected to be antigenically distinctive. Although the achievement of proper control in this sense is necessarily difficult, it has been accomplished in some measure in current experiments in which supernatants of whole-mouse homogenates (mouse decapitated, bled, washed, and shaved; intestine stripped and washed; carcass finely minced with scissors and homogenized in Waring blendor) of the respective tumor-host strains were used to absorb antiserums against the S91 and B16 tumors. The absorbed antiserums, in reaction with their respective homologous tumor preparations, produced definitive precipitates, presumably of antigenically distinctive tumor components.

These findings have been further confirmed in preliminary studies with the Ouchterlony (*16*) double-diffusion agar technique. Antitumor serums, absorbed with supernatants of "whole-mouse homogenates" of their respective tumor-host strains and simultaneously confronted with antigenic preparations of normal mouse tissues and of tumors, show precipitation lines only with the tumors.

As noted in the introductory comments, many studies have failed to reveal antigenic specificity in tumors. Korngold and Van Leeuwen (*17*) have suggested that the explanation for these failures may be due, in part, to the fact that some antigens of a tumor compete more strongly for antibody-forming sites than others do. The production of antibodies against tumor-specific antigens may thus be inhibited by "stronger" antigens which are common to both normal and neoplastic tissues. This view receives support from studies of the effect of injecting simultaneously two distinct purified soluble-protein antigens into chickens (*11*). The

"stronger" antigen both reduces and delays the immune response to the "weaker" (*cf.*, also, *18* and *19*).

Failure to demonstrate antibody responses to specific neoplastic antigens may result also from the use of adjuvant techniques and of multiple injections of antigens administered over a long period. A second injection of mixed antigens may enhance the competitive suppression of antibody response to the weaker antigens. This interpretation is strongly suggested by the results of current studies (Abramoff, unpublished data) in which mixtures of purified soluble-protein antigens are administered to birds in a multiple series of injections over a period of several weeks.

Furthermore, it may be suggested that, although multiple injections and the use of adjuvants are usually effective in enhancing antibody titers, the use of these techniques may decrease the specificity of the antibody response. An antitumor serum resulting from a series of injections might thus lack specificity to the extent that it would cross-react completely with antigens of the tumor host, even though the tumor itself had distinctive antigenic components. The reasonableness of this view is attested by numerous studies (*20–22*) which show that multiple injections tend to reduce the specificity of antiserums produced against both soluble and particulate antigens.

In the present study the successful demonstration of tumor-specific antigens thus may be due in large measure to the use of the chicken, which produces appreciable quantities of antibodies after only a single injection of antigen. Further studies now are being directed toward the separation of the tumor-specific antigens with a view to reducing the presumed competitive effects of nonspecific tumor components. Proper fractionation of the tumor (*cf. 17*) may permit single injections of a tumor component which will elicit specific antitumor antibodies of high titer.

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Comparison of the Number of Breast-Cancer Deaths Observed in Relatives of Breast-Cancer Patients, and the Number Expected on the Basis of Mortality Rates [1, 2]

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Summary

Grandmothers, mothers, aunts, and sisters of women with breast cancer have had breast cancer with a frequency which is significantly greater than that of women in a similar age range either in the general population or in 2 sets of selected control samples. This excess cannot be entirely environmental in origin, since it is found almost to the same extent in both paternal and maternal grandmothers and aunts. It cannot be attributed to biased selection or to chance distribution of the trait through the population, since the excess is significantly greater than can be accounted for by either of the above factors. Better recall by women with breast cancer, whose relatives are similarly affected, has no bearing on this excess, since the entire family of both control and breast-cancer samples was thoroughly investigated. The ratio of observed-to-expected number of breast cancers is higher in unmarried than in married aunts and sisters. The fact that the presumably genetic factor responsible for this excess of breast cancer is enhanced to the greatest degree in childless women shows the interaction of genetic and extrinsic factors.—**J. Nat. Cancer Inst. 22: 927–951, 1959.**

A number of studies, by Jacobsen of Denmark (*1*), Penrose *et al.* of England (*2*), Woolf (*3*), and Anderson *et al.* (*4*) in the United States, have been made in an endeavor to answer the question of whether or not breast cancer in the human being has a genetic basis. A similar study was begun by the author, in 1946, on a sample population from the State of Ohio. The present paper deals with evidence obtained by the use of mortality rates to determine the number of breast cancers expected in sample populations related to 1) a proband with breast cancer—the group of relatives designated as P1; 2) a proband with cancer in some organ other than the breast—this group of relatives designated as P2; and 3) a proband who had, at the time of interview, no symptoms or signs of breast cancer—this group of relatives designated as P3. The material

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was collected from June, 1946, through 1952. Not enough breast-cancer cases were found in any 1 year to form a sufficiently large sample.

Method of Collecting Data

The manner in which the P1, P2, and P3 probands were selected was as follows: The 295 P1 probands with breast cancer were chosen from women, in the hospitals of Columbus, who had had surgery for proved breast cancer and who were either in the hospital at the time of the operation or in the Columbus Cancer Clinic where follow-up procedures for patients from all hospitals were carried out. Anderson *et al.* (*4*) refer to the difficulties in securing an unbiased set of both probands and controls, and state that the use of probands from more than one hospital increases the difficulties in securing an unbiased selection. They used only probands who had come to the University Hospital Tumor Clinic either as referrals or as primary patients.

Anderson *et al.* quote the author (p. 38, *5*) as saying, "The family history was unknown to the investigator at the outset of the interview, and hence no selection was possible whereby women with a great deal of breast cancer in their relatives could be chosen." They then comment that if a hospital has gained a reputation for especially effective treatment for some type of cancer, and ". . . if any of the reasons for referral to a clinic serving as a source of breast cancer propositae [the same as probands] are related to the presence or absence of breast cancer in their relatives, this could be a very serious source of bias and might invalidate the conclusions of an otherwise carefully planned study." This source of bias was avoided by taking the probands from all the hospitals in the city. No woman was used as a proband if she had already been listed as a relative in the family of another proband, an event which occurred only once in collecting the data.

It should be made clear at this point that all the probands used in this study were residents of Columbus and its immediate environs; they were not referred here from other parts of the State, but came to 1 of the 6 hospitals in the city because they lived in Columbus, and in the great majority of instances probably could not afford to travel elsewhere for operative treatment. If any bias exists, it would be in the direction of obtaining probands and a group of relatives of such socioeconomic status that they would have *less* breast cancer than the average. Those patients with a desire and money enough to hunt for treatment at nationally known cancer centers would not have been encountered in this study. They are the very patients of the higher socioeconomic range in which breast cancer is said to be most prevalent.

Only white women were interviewed, and only those could be used as probands who had at least the desired information on their parents and their sibs, and who were willing to cooperate. This eliminated those women who were adopted, those who had relatives abroad who could not be contacted, those who were too ignorant to answer questions intelligently, and those who resented being questioned at all.

Criticism has been advanced that a very real bias may come from the elimination of the women indicated, and that ". . . *ideally* an examination should be made of *their characteristics* as compared to those of the women who did participate in the study, to determine possible differences between the two, and, therefore, the biases inherent in eliminating them." [Author's italics.] It is in the *relatives* of these women that the interest lies, not in the probands themselves. Had the information on the relatives been obtained, the probands naturally would not have been eliminated. Such a criticism demands obtaining the unobtainable in order to estimate the bias engendered through not obtaining them! Approximately 45 women were eliminated for the reasons indicated, or 13 percent of the total number of names secured.

The 300 P2 probands were both men and women in the hospitals of Columbus and in the same cancer clinic where the P1 probands were selected. They matched almost ideally the P1 probands in socioeconomic condition, intelligence, knowledge of their family, etc. Some were excluded for the same reason that P1 probands were. The cancers which they had were diverse in nature, though the majority were cervical, rectal, gastric, and skin cancers. Males could be accepted as probands, since it was not the probands but the relatives in whom the interest lay.

The reason for collecting probands with cancer other than breast cancer was that such a group would enable one to determine whether the factors for cancer were site-specific or for malignancy in general. If site-specific, there should be no more breast cancer in P2 relatives than in the general population; but if the factors inducing cancer were for any malignancy regardless of site, the relatives of P2 probands should exhibit as much breast cancer as the relatives of P1 probands.

The 246 P3 probands were selected in various ways and did not constitute as ideal a control population of probands as desired, though, as shown later, their relatives had breast and other cancers in numbers not significantly different from that of corresponding persons in Ohio. Some of them were in the hospital for conditions other than cancer and were from the group of ward patients, thus matching the P1 probands in socioeconomic status. Others were obtained from among the friends of these ward patients who were visiting the proband, either in the hospital or in her home when the interview was taken in the home. Still other control probands were obtained from volunteers who responded to an appeal in the Columbus newspapers for white women, between the ages of 40 and 70, who knew something of their family history, and who had no known cancer of any kind. They were somewhat higher in socioeconomic status than the average P1 proband, though not markedly so. They were rejected as volunteers if they said they had come because there was no known cancer in their family. They were accepted if they stated that there was cancer in their family (there were 2 such P3 probands taken). P3 probands, who had a higher socioeconomic status than the P1 probands, were obtained from women who worked on the campus or whose husbands worked on the campus. This latter group would affect the

results in only one way however: by minimizing any difference that might exist between P1 and P3 relatives as far as frequency of breast cancer was concerned, since breast cancer is unduly frequent among those of higher socioeconomic status, whatever the underlying etiological factor may be. This bias would occur, provided P3 relatives had the same socioeconomic status as P3 probands, which was by no means always true. The P3 probands were all women who matched in age, woman for woman, one of the P1 probands.

The distribution of the P3 probands was as follows: 126 (51.2%) women selected by the author with no knowledge of their family background, but with the same socioeconomic and cultural levels as P1 probands; 84 (34.2%) women volunteering after an appeal had appeared in Columbus papers; 36 (14.6%) university women cooperating after a personal appeal to them.

Although the third group constituted 14.6 percent of the P3 probands, their relatives were only 8.7 percent of the group of P3 relatives, exclusive of cousins who are the least important genetically of the relatives studied. The second group made 34.2 percent of the probands, and their relatives, exclusive of cousins, made 36.5 percent of the relatives. The first group, comprising 51.2 percent of the probands, was represented by 54.8 percent of the relatives. The third group, against which criticism might be leveled with most justification as far as probands were concerned, formed a very minor group of relatives. Moreover, the number of breast cancers found in the relatives of the 3 groups of P3 probands was quite in proportion to the number of relatives each group contributed. They were, therefore, treated as a homogeneous group of P3 relatives.

Again, criticism has been advanced that no ill persons should have been admitted as P3 probands, and the stipulation should not have been made that such women should have no known signs of breast cancer. With respect to the first of these criticisms, one must realize that, in order to obtain a sufficiently extensive sample for P3 probands, a very large group of women would have to be examined thoroughly in order to rule out those with any type of illness. This would require a battery of experts to administer physical, mental, chemical, endocrinological, and radiographic examinations, a time-consuming and an expensive project. It would also require the cooperation of a group of presumably well women, far greater than the number finally needed, since these women, matching in age the P1 probands with breast cancer, would be in the higher age ranges where some type of illness, such as arthritis, goiter, diabetes, cardiovascular disease, etc., would be very common. The investigator has to be realistic about the plan of the study. Moreover, no association has been shown between these conditions in a woman and breast cancer in any of her female relatives.

The second criticism involves the problem of bias, because the woman who had no breast cancer would be less likely to recall the existence of breast cancer in her family, or, on the other hand, the woman who had breast cancer among her relatives might be more anxious to volunteer.

This brings up the problem of "recall." The argument is advanced repeatedly that the woman with breast cancer will be more likely to recall other relatives who have had operations on the breast than the woman who does not have breast cancer. Hence, on this basis alone, one would find more breast cancer reported in families of P1 probands than in families of P2 or P3 probands.

It must be emphatically stated that, in this study, the problem of recall does *not* bias the results, as it may well do in some studies reported in the past, in which the investigator checked the accuracy of the proband's statement if she said that a relative *had had cancer*, but did not check her statements that a relative did not have cancer. In such a study, one is relying on the proband completely, when cancer is not reported, and checking only when it is reported. *The history secured from the proband in this study did not constitute the data on which the tables in this paper were based.* Every statement concerning relatives was checked and cross-checked, as will be described in the next paragraph, so that many relatives with breast cancer were uncovered whom the proband had never mentioned. This was true of P1, P2, and P3, so that *better recall of P1 probands does not bias the data.* Not only did the problem of better recall, by those who knew they had breast cancer, not enter the picture, but many of the P1 probands, having been assured by their surgeons that they had only a benign tumor, did not know that they themselves had breast cancer.

Identical interviews were held with P1, P2, and P3 probands about their personal medical history, their grandparents, parents, aunts, uncles, sibs, children, and first cousins. *Intensive investigation of these relatives was then undertaken to check the accuracy of the information furnished by the proband and to add much that the proband had not mentioned.* Death certificates of all deceased relatives, regardless of whether the proband or other relatives had stated that cancer was the cause of death, were obtained whenever possible from all States and also from foreign countries. No cancer reported by the proband or by any of her relatives was accepted as such unless confirmed by the death certificate. All living relatives within the scope of the study and the persons through whom they were related to the probands were either visited or written to for firsthand information. This investigation provided correct ages, dates of birth and death of many deceased relatives the probands had but imperfect knowledge of, and furnished names of other relatives within the scope of the study that the proband had forgotten. The number of relatives added to those furnished by the proband varied in different families from none to over 100. The result of this investigation showed that the sample of 300 P1, P2, and P3 probands first interviewed had accurate knowledge of only one fourth of the cancer cases in the entire group of relatives studied and accurate knowledge of only one half of the cancer cases even in their parents and sibs.

Before the data are presented, several other possible sources of bias will be considered. Among women whose names were secured as potential P1

probands, only those who cooperated could be included. If this cooperation depended on the fact that a large proportion of the probands were aware of breast cancer cases in close relatives, dead or alive, and cooperated because of that knowledge, the sample would probably be biased in favor of an unduly large number of familial cases. A survey of the histories of the 295 women who did cooperate was made with the following results: 91 probands did not report any type of cancer in their relatives, but breast cancer was found in 18 of these families; 105 P1 probands reported cancers of other types but no breast cancer, though breast cancer was found in 15 families; 99 P1 probands reported breast cancer in their relatives, but, in 17 instances, the breast cancers were not verified, although other relatives not reported as having cancer were found to have breast cancer. In 23 instances the report on breast cancer by the proband was verified and no new cases were found, and in 59 instances, no breast cancer was found. This is not to say that in some of these 59 instances there was no breast cancer; it merely means that no verifiable record which could be used in the mortality tables was obtained. It would seem that in only 23 of 295 probands was the cooperation based on accurate knowledge.

A further possible bias arises because, in all 3 sample populations studied, the information on each group of relatives was not complete. Among the 590 P1 grandmothers, information was complete on 51 percent. If the information on the 49 percent of grandmothers had been obtained, it might or might not have materially modified the results. Similarly, on 52.3 percent of P2 grandmothers and 37.2 percent of P3 grandmothers, information was not obtained. On the other hand, information was complete on 87.3 percent of P1 mothers, 86.5 percent of P2 mothers, and 90.9 percent of P3 mothers who had lived to the age of 30; and on 93.3, 95, and 97.8 percent, respectively, of sisters of P1, P2, and P3 probands. Since similar results from grandmothers with incomplete coverage are obtained from mothers and sisters with almost complete information, it is not unreasonable to assume that the sample of grandmothers was fairly representative of the 3 populations.

It has been pointed out that some P1 probands were interviewed while they were still in the hospital following an operation, and that therefore they would be the last members in their families to be diagnosed, while those P1 probands who were interviewed at the Columbus Cancer Clinic, subsequent to their operation, were not necessarily the last diagnosed in their families; other relatives might have become affected with breast cancer after the operation on the proband. It was suggested that the data for the 2 groups of probands be presented separately to eliminate any bias. This has not been done in full, since a preliminary examination of the material showed little difference between the 2 groups (table 1).

There is the bias which is peculiar to the selection of samples when one cannot study the entire population. A bias is said to exist, when a sample is collected from the population, because of a particular trait which is present. If there are, for example, 2 sisters with breast cancer in family A,

TABLE 1.—*Effect of time of interview on frequency of breast cancer in families of P1 probands*

P1 probands	Time of interview with P1 proband	
	At time of operation	After operation
Number of P1 probands interviewed	145	150
Number of probands with no breast cancer reported either by the proband or a relative	96	100
Number of remaining P1 families	49	50
Number of breast-cancer patients in remaining P1 families	82	79
Found or verified through death certificates	57	58
Reported by family, but not verified by records obtained or because of lack of records	25	21

one could find family A through either of the sisters, whereas in family B with only 1 sister affected, there is only 1 chance of finding the family. If one were to miss the 1 sister, that family would not come under observation. Families with multiple cases are therefore said to be represented in selected samples with greater frequency than they occur in the population. Perhaps the bias should be stated in this manner: Families with multiple cases *may* be represented in selected samples with greater frequency than they occur in the population. There is no mathematical formula which says that they *must* be more frequently represented in selected samples.

The probability of such a bias certainly exists but not necessarily under all circumstances. For example, if the probands were obtained through examination of death certificates over a period of years, for 1 State, and if 2 sisters or mother and daughter had succumbed to breast cancer in that State and in the years under investigation, the family could be ascertained twice, either through finding certificates for both sisters or for mother and daughter. Such a bias would not exist if one chose only living probands from a given region, and if the other relatives with breast cancer had lived or died *outside* the locality being investigated. Such families could not possibly be ascertained more than once. The only possibility of encountering families with multiple cases would arise if several living relatives were treated, in the same locality and in the period of years under study. There thus exists a possible theoretical, and an actual, bias, and it is the latter which actually prejudices the results. Both will be discussed.

To assess the degree of actual bias in a given sample, one must know the population values. It is precisely these latter values that are unobtainable. The percentage of families in the general population that have 1 or more additional cases of breast cancer, within the circle of relatives of grandmothers, mothers, aunts and sisters, is unknown. Hence, it cannot be stated that the percentage of families in this study, which have 1 or more cases of breast cancer in addition to that of the proband, is too great to be attributed to chance sampling. The data of this study reveal that among the female relatives of 295 P1 probands, exclusive of cousins,

there were no additional verified cases of breast cancer in 222 families or 75.3 percent; there was 1 additional case in 56 families or 19 percent; 2 additional cases in 12 families or 4 percent; and 3 additional cases in 5 families or 1.7 percent. It is impossible to say whether these values are significantly in excess of general population values. It can only be said that in the 73 families with multiple cases, only 1 family was ascertained twice. In this instance the second sister with breast cancer was *not* used as a proband, and the family was used only once.

The argument is advanced, however, that it is not the observed but the theoretical bias which must be taken into consideration. The theoretical bias has been dealt with as follows: If 56 families were found in which there was a case of breast cancer in addition to that in the proband, and if families with 2 cases are likely to be found twice as often as they would be if they had only 1 case, it is obvious that 28 of these families were found because they did have 2 cases, and they should, therefore, be excluded from the computations. The proband was, of course, not included in any analysis, and exclusion of the second case in 28 families would leave 28 to be included. Similarly, the 12 families with 2 extra cases besides the proband would have been found 3 times as often as they occurred in the population, so that only 4 such families should have been found; and the 5 families with 3 additional cases would have been found 4 times as often, leaving only 1.25 families to be included. Excluding those families which were *theoretically* found unduly often, we have the following results:

$56 \div 2 = 28 \times 1$ cases to be entered in tables	28
$12 \div 3 = 4 \times 2$ cases to be entered in tables	8
$5 \div 4 = 1.25 \times 3$ cases to be entered in tables	3.75
Total number of cases expected on basis of no bias	39.75
Total number of cases expected on basis of age-specific, proportionate death rates in 1,304 relatives exclusive of cousins	28.22

$\chi^2 = 4.41$ (d.f.) $= 1$, $P > .03$

The actual number of breast cancers found in these 73 families, in addition to the probands, was 95. It would appear from these figures that even with the elimination of 55.25 cancer cases, which might have been included due to biased ascertainment, the excess of breast cancers was still enough to make it very unlikely that this sample of P1 relatives had the same amount of breast cancer as was present in the general population or in P2 or P3 relatives.

Age-specific, proportionate death rates rather than mortality rates were used to give the expected number of breast cancers in the preceding calculation, since the former rates are more nearly applicable to the period of death of the relative observed. The former rates give an expected value of 4.5 for P1 grandmothers (table 5) in contrast to 7.4 (table 2) based on the latter rates. Among P1 mothers and aunts, 23 and 25 percent, respectively, had died before 1914, and 75.6 and 68.8 percent, respectively, had died before 1940. For this reason, age-specific, proportionate death rates were used to determine the expected value of 28.22 used.

It is recognized that death certificates are not wholly accurate. There is no reason to assume, however, that the degree of accuracy differed among the relatives of P1, P2, and P3 probands. Failures in diagnosis and recording were just as likely to happen in grandparents and parents of the control probands as in the grandparents and parents of the P1 probands. Only relatives were used whose death certificates had been obtained, for, although the accurate age of death might be accessible, the cause of death would not be known from an official record. Moreover, it is the death certificates on which the mortality rates used in the study are based.

In part of this study, reported elsewhere, the vital statistics for Ohio were used, since they were available, and 90 percent of the deaths of the relatives occurred in Ohio. The use of mortality rates in determining the expected number of breast cancer cases in these 3 sets of relatives was impossible without turning to the mortality statistics for the whole of the United States—mortality rates for Ohio only were not available either in Ohio, or in the published records of mortality statistics from United States reports. The records for the whole country may not be strictly applicable to an Ohio population for a number of reasons, but since the same standard was to be applied to P1, P2, and P3 sample populations, it was felt that the conclusions would be valid.

Use of Mortality Statistics in This Study

The mortality rates for breast cancer have not changed materially during the past few decades; hence it was possible to select the 1940 mortality rates for breast cancer in the United States and apply them to the present study, for mothers, aunts, sisters, and cousins. Rates for years earlier than 1940 were used for grandmothers who had died in earlier periods. First, the rates for single, married, widowed, and divorced women in age groups from 25 to 34, 35 to 44, 45 to 54, and in quinquennial age groups after that, were listed in the 1940 United States Census Reports (*6*). Second, the rates for nonwhite and white women in the same age groups were given. No table contained the combined age group, marital status, and color separation. Hence the following assumptions were made, and it is felt that they do not invalidate the conclusions: Breast cancer is not common before 30 years, the age which all relatives in this study had to reach before they were coded. Sisters, cousins, or aunts who died, or were living, at that age, were not included. Hence the death rate for breast cancer among women 25 to 34 years old was used as if it were the rate for women 30 to 34 years old. This would merely make the expected number of breast cancers in that age group slightly higher than they should be, thus minimizing the difference between the observed and the expected number in P1, if the observed number tended to be higher than the expected.

The next assumption dealt with the rates for single and ever-married women. In the study, no inquiry was made as to whether the grandmothers, mothers, aunts, sisters, and cousins were married and living with

their husbands at the time of death or at the time of contact, or whether they were widowed or divorced. If they had been married at any time in their lives, they were listed as married. This meant that the mortality rates for the ever-married population in the United States had to be computed on the basis of the number of women and the number of breast cancers in the combined categories of married, widowed, and divorced. Since the United States Census Reports did not give data on how many single women were white or nonwhite, it was assumed that any nonwhite woman who had attained the age of 30 had been married, and, therefore, the entire rate for single women would apply to white women. This would make the number of expected breast cancers among single women a little too high for purposes of comparison with the single women in this study, and, correspondingly, make the number of expected breast cancers among the married women a little too low. Again, the technique was applied to all 3 sample populations, and all 3 were subjected to the same bias.

The United States mortality rates for breast cancer among divorced and widowed women are higher than those for married women. The reason for not separating the ever-married women, in the study, into married, widowed, and divorced is as follows: For example, a women whose breast cancer began at the age of 45, when she was married, might become either divorced or widowed before the age at which she died of breast cancer. (There have been instances when men divorced their wives because they developed breast cancer.) The marital status of such women at the time of death would in no way correspond to their status at the time they developed breast cancer, which is the point of interest. Similarly, a woman who had remained unmarried and developed breast cancer during that time, and then married later (as one of the probands did), would be listed as married at the time of her death from the cancer, though it was her single status that had doubtless contributed to her breast cancer. Unless the widowed or divorced status can be correlated with the woman who has fewer children than the woman who has remained married, or correlated with whether she developed the cancer after or before the change in marital status, the author is of the opinion that such categories are meaningless. It would seem highly unlikely that the decree of a divorce court or the death of a husband could induce breast cancer, unless there can be shown to be some correlation between sexual life and breast cancer. Since the unmarried woman had no more breast cancer than the childless, married woman, in this study, the role of sexual activity in suppressing breast cancer remains unproved in this sample.

Method of Computing the Number of Person-Years of Risk

All aunts, sisters, and cousins were considered as having entered the study at the age of 30, since none under that age was coded. In the P1 aunts and P1, P2, and P3 sisters, the exact age, in years and months, at which they dropped from the study either by death or because the author had no further contact with them after the interview was known and put

on the coding card. The age of the P2 and P3 aunts and all cousins was coded in quinquennial age groups, and the computations were based on the assumption that they had reached the mid-point of the age group. The mothers were assumed to have entered the study at the age they had reached in years and months when the proband, who had brought them into the study, was born. Thus, if the mother were 34 years and 7 months old at the birth of the daughter who later developed breast cancer, she was credited with only 5 months of living experience between the ages of 34 and 35. If she died at the age of 69 years and 11 months, she was credited with a full year of living experience between 68 and 69, and with 11 months between 69 and 70. Similarly, the paternal grandmother was listed as entering the study at the age she had reached when the father of the proband was born, and the maternal grandmother at the age she had reached when the mother of the proband was born. The number of person-years was computed for each set of relatives in each of the 3 sample populations. In all groups except grandmothers and mothers, the number of persons ever present in any age group rises steadily from the bottom of the table to the top. This is not true of grandmothers and mothers, because not all of them entered the study at the age of 30.

The 1940 rates had been used for mothers, sisters, aunts, and cousins, but the rates for grandmothers had to be based on some year earlier than 1940. It was found that 43.5, 50.5, and 44.5 percent of the grandmothers in P1, P2, and P3, respectively, had died before the year 1910. The rates used were for 1914 (7), though they were not wholly satisfactory, since the rates were for white and nonwhite combined. The rates for the nonwhite were lower than those for the white, and the nonwhite also constituted a relatively small proportion of the population. Since all P1, P2, and P3 grandmothers were to be compared with the same standard, the conclusions may be accepted in general, though the specific numbers may be incorrect.

The expected number of breast-cancer deaths is based on the mortality rates for 1914, given in table 2. Seventeen breast cancers were listed on the death certificates of the 301 P1 grandmothers for whom records were found; only 7.41 breast cancers were expected. X^2 in all these tables means that the Yates correction has been applied. X^2 is significant, $P<0.001$.

Table 3, for 286 P2 grandmothers, shows a greater number of breast-cancer deaths expected (6.71) than were found (2), though the X^2 values are not significant at the 5 percent level. These grandmothers, as well as those in P1 and P3 shown in another part of this study, had about the same amount of cancer of all kinds as the Ohio population had, of similar age range and decade of death. In 306 P3 grandmothers (table 4), there was somewhat less breast cancer than expected (3 cases found and 7.63 expected), but again the difference was not significant.

It has been suggested that, since most of the grandmothers were deceased, age-specific, proportionate death rates should be used rather than mortality rates. This has been done, and the results are given in table 5.

TABLE 2.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in 301 P1 grandmothers**

Age group	Number of persons ever present in group	Number of person-years	United States mortality rates from breast cancer per 100,000 women	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	248	1115. 00	2. 9	0. 03	0
35–39	277	1318. 50	14. 1	0. 19	0
40–44	288	1391. 75	25. 7	0. 36	0
45–49	285	1395. 25	42. 3	0. 59	0
50–54	278	1370. 00	51. 2	0. 70	4
55–59	269	1314. 00	69. 0	0. 91	1
60–64	258	1231. 00	68. 8	0. 85	4
65–69	234	1086. 00	85. 7	0. 93	1
70–74	200	889. 75	103. 1	0. 92	2
75–79	153	639. 75	120. 4	0. 77	2
80–84	103	413. 50	163. 0	0. 67	0
85+	53	269. 00	181. 5	0. 49	3
			Totals	7. 41†	17†

*Before 1910, 43.5 percent of grandmothers had died.
†$X^2 = 11.43$ (d.f. = 1), $P<0.001$.

Ohio death data were used. The results were obtained by separating the persons into 13 quinquennial age groups, beginning with 30 to 34 years and ending with 90+ years, and into 6 time periods, combining all those who died before 1910 and using the 1910 death data of Ohio for this group. The other periods were by decades from 1910 to 1950, and the years 1950–52. The average number of deaths and the average number of deaths from breast cancer for each decade (all relating to white women only) were computed and used in deriving the values in table 5.

TABLE 3.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in 286 P2 grandmothers**

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	237	1036. 75	0. 03	0
35–39	264	1237. 50	0. 17	1
40–44	274	1373. 00	0. 34	0
45–49	269	1308. 00	0. 55	0
50–54	259	1272. 25	0. 65	0
55–59	254	1237. 50	0. 85	0
60–64	240	1153. 25	0. 79	0
65–69	215	1000. 50	0. 86	0
70–74	181	800. 75	0. 83	0
75–79	138	574. 25	0. 69	0
80–84	88	325. 75	0. 53	1
85+	45	233. 00	0. 42	0
		Totals	6. 71†	2†

*Before 1910, 50.5 percent of these grandmothers had died.
†$X^2 = 2.69$ (d.f. = 1), $P = 0.20$ to 0.10.

TABLE 4.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in 306 P3 grandmothers**

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	231	1103. 75	0. 03	0
35–39	272	1318. 00	0. 19	0
40–44	290	1437. 00	0. 37	0
45–49	293	1459. 75	0. 62	0
50–54	288	1426. 00	0. 73	0
55–59	279	1321. 25	0. 91	1
60–64	254	1215. 00	0. 84	0
65–69	234	1099. 75	0. 94	0
70–74	203	916. 75	0. 95	1
75–79	165	714. 25	0. 86	0
80–84	110	430. 75	0. 70	1
85+	61	269. 25	0. 49	0
		Totals	7. 63†	3†

*Before 1910, 44.4 percent of these grandmothers had died.
†$X^2 = 2.27$ (d.f. = 1), $P = 0.20$ to 0.10.

Tables 6, 7, and 8 give the expected and the observed number of breast cancers in P1, P2, and P3 mothers. Table 6 carries the mortality rates for ever-married women for 1940. The number of breast-cancer deaths observed in P1 mothers was double that expected for 1940 rates (11 to 5.6). $X^2 = 4.38$, $P > 0.02$.

In P2 mothers the number of observed breast cancers, 4, is a little less than the expected number, 5.88 (table 7). $X^2 = 0.33$, $P > 0.50$. Similarly, among the 221 P3 mothers, 3 breast cancers were found and 4.58 were expected. $X^2 = 0.26$ and $P > 0.50$. Thus, P2 and P3 mothers had the amount of breast cancer which the rest of the population of Ohio of similar age experienced, but P1 mothers had twice as much.

Since the mortality rates for breast cancer among single women are higher than among ever-married women, aunts, sisters, and cousins were divided into single and ever-married categories. The observed breast-cancer deaths were just about twice the number of the expected, 36 and

TABLE 5.—*Observed and expected numbers of breast cancer in P1, P2, and P3 grandmothers, based on age-specific, proportionate death rates for white females in Ohio from 1910 to 1952, by decades and by quinquennial age groups*

Population	Number with death certificates	Number of breast cancers listed on death certificates			Number expected on age-specific proportionate death rates	X^2 (d.f. = 1)	P
P1 grandmothers	297	17	8*	9†	4. 5	32. 49	<0. 0001
P2 grandmothers	283	2	1*	1†	4. 4	1. 06	=0. 30
P3 grandmothers	302	3	1*	3†	4. 7	0. 31	>0. 50

*Additional breast cancers but not listed as such on death certificate.
†Additional breast cancers but no death certificate found.

TABLE 6.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in 255 P1 mothers*

Age group	Number of persons ever present in group	Number of person-years	U.S. mortality rates for breast cancer for ever-married women per 100,000* for 1940	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	205	918.00	5.229†	0.05	0
35–44	251	2305.50	18.338	0.42	0
45–54	242	2323.50	44.589	1.04	2
55–59	219	1048.75	61.935	0.65	1
60–64	198	951.50	82.395	0.78	1
65–69	182	824.00	92.562	0.76	1
70–74	149	620.75	114.580	0.71	3
75–79	99	425.75	140.546	0.60	1
80–84	65	223.75	175.434	0.39	2
85+	27	92.50	211.000	0.20	0
			Totals	5.60‡	11‡

*It has been assumed that all nonwhite women of 30 years or more have been married.

†This is the rate for age group 25 to 34 but has been used as the rate for the 30 to 34 group. (These two footnotes apply to all data dealing with ever-married relatives in P1, P2, and P3.)

‡$X^2 = 4.38$ (d.f. = 1), $P = 0.05$ to 0.02.

18.27, in the ever-married P1 aunts (table 9) and 3.8 times the expected number, 7 and 1.84, in single P1 aunts (table 10). X^2 is significant at the 0.0001 level in table 9 and at 0.001 in table 10. Tables 11 and 12 show the expected and the observed number of breast cancers in P2 ever-married and single aunts, respectively. The expected number was greater, though not significantly, in the ever-married aunts (17.44 to 10), and close to the observed value in single aunts (1.98 to 1). The two X^2 values were 2.82 and 0.12, respectively, with P values of >0.05 and >0.70.

TABLE 7.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in 257 P2 mothers*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	211	946.00	0.05	0
35–44	254	2350.00	0.43	0
45–54	247	2365.75	1.05	0
55–59	229	1089.50	0.67	0
60–64	213	1011.00	0.83	1
65–69	190	846.00	0.78	0
70–74	149	655.00	0.75	2
75–79	107	438.50	0.62	0
80–84	68	231.25	0.41	1
85+	30	139.50	0.29	0
		Totals	5.88*	4*

*$X^2 = 0.33$ (d.f. = 1), $P = 0.70$ to 0.50.

TABLE 8.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in 221 P3 mothers*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	177	752. 50	0. 04	0
35–44	216	1965. 50	0. 36	0
45–54	208	1988. 00	0. 89	1
55–59	189	898. 00	0. 56	0
60–64	171	792. 00	0. 65	0
65–69	140	628. 75	0. 58	0
70–74	105	476. 00	0. 55	0
75–79	82	336. 50	0. 47	2
80–84	53	184. 50	0. 32	0
85+	19	77. 50	0. 16	0
		Totals	4. 58*	3*

*$X^2 = 0.26$ (d.f. = 1), $P = 0.70$ to 0.50.

Tables 13 and 14 show the expected and the observed number of breast cancers in ever-married and single P3 aunts. The expected number, 14.31, was twice the value of the observed, 7, in the ever-married aunts, but the difference is not significant at the 5 percent level, $P>0.05$. The reverse was true in the single aunts, but the difference is not significant, $P>0.50$. Again, P1 aunts have significantly more breast cancer than would be expected, while P2 and P3 aunts do not differ significantly from the expected values.

Tables 15 and 16 deal with ever-married and with single P1 sisters, respectively. The observed value 11 was 2.5 times the expected value

TABLE 9.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P1 aunts*

Age group	Number of persons ever present in group	Number of person-years	U.S. mortality rates for breast cancer for white ever-married women per 100,000*	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	836	4157. 75	5. 229†	0. 22	0
35–44	824	8133. 25	18. 338	1. 49	4
45–54	792	7646. 00	44. 589	3. 41	7
55–59	713	3449. 00	61. 935	2. 14	2
60–64	657	3144. 25	82. 395	2. 59	4
65–69	584	2652. 50	92. 562	2. 46	8
70–74	476	2040. 50	114. 580	2. 34	9
75–79	333	1286. 25	140. 546	1. 81	0
80–84	192	673. 25	175. 434	1. 18	1
85+	83	297. 25	211. 000	0. 63	1
			Totals	18. 27‡	36‡

*In computing this rate it is assumed that all nonwhite women are married by the age of 30.
†The rate covers 25 to 34 years of age but has been used as the rate for 30 to 34 years.
‡$X^2 = 16.61$ (d.f. = 1), $P<0.0001$.

TABLE 10.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P1 aunts*

Age group	Number of persons ever present in group	Number of person-years	U.S. mortality rates for breast cancer for single women per 100,000*	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	55	271.00	10.234†	0.03	0
35–44	54	536.75	25.965	0.14	0
45–54	52	506.00	76.943	0.39	0
55–59	49	237.50	122.716	0.29	0
60–64	45	200.00	134.532	0.27	2
65–69	35	157.00	151.532	0.24	0
70–74	28	105.25	168.871	0.18	1
75–79	15	61.00	213.456	0.13	2
80–84	9	33.00	301.367	0.10	1
85+	5	30.75	213.126	0.07	1
			Totals	1.84‡	7‡

*Based on assumption that all single women who have reached the age of 30 are white.
†This rate is for 25 to 34 years of age, but it has been taken as the rate for 30 to 34 years in these tables.
‡$X^2 = 12.21$ (d.f. = 1), $P<0.001$.

(4.47) in married sisters, and in the single sisters, the observed value 3 was 8 times that expected (0.37). The difference is significant in table 15, with P being less than 0.01, but, in table 16, X^2 is not shown because of the small observed and expected frequencies.

Tables 17 and 18 deal with the sisters of the P2 population and, here, observed and expected values were very close: 5 and 5.04 for the married, and 1 and 0.88 for the single sisters.

Tables 19 and 20 show that the observed and the expected values for P3 sisters were also close: 3 and 2.79 for married, and 2 and 0.59 for single sisters.

TABLE 11.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P2 aunts*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	811	4017.50	0.21	0
35–44	796	7785.00	1.43	0
45–54	758	7347.50	3.28	4
55–59	699	3362.50	2.08	0
60–64	646	2995.00	2.47	1
65–69	552	2467.50	2.28	1
70–74	435	1850.00	2.12	1
75–79	305	1217.50	1.71	1
80–84	182	665.00	1.17	2
85+	84	325.00	0.69	0
		Totals	17.44*	10*

*$X^2 = 2.82$ (d.f. = 1), $P = 0.10$ to 0.05.

TABLE 12.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P2 aunts*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	47	232. 50	0. 02	0
35–44	46	457. 50	0. 12	0
45–54	45	435. 00	0. 33	0
55–59	43	200. 00	0. 25	0
60–64	37	182. 50	0. 25	0
65–69	36	170. 00	0. 26	0
70–74	32	147. 50	0. 25	1
75–79	27	107. 50	0. 23	0
80–84	16	65. 00	0. 19	0
85+	10	40. 00	0. 08	0
		Totals	1. 98*	1*

*$X^2 = 0.12$ (d.f. = 1), $P = 0.80$ to 0.70.

TABLE 13.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P3 aunts*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	668	3317. 50	0. 17	0
35–44	659	6400. 00	1. 17	0
45–54	625	5980. 00	2. 67	0
55–59	561	2685. 00	1. 66	0
60–64	513	2405. 00	1. 98	1
65–69	449	2050. 00	1. 89	2
70–74	371	1577. 50	1. 81	3
75–79	260	995. 00	1. 40	1
80–84	138	527. 50	0. 93	0
85+	73	297. 50	0. 63	0
		Totals	14. 31*	7*

*$X^2 = 3.31$ (d.f. = 1), $P = 0.10$ to 0.05.

TABLE 14.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P3 aunts*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	85	420. 00	0. 04	0
35–44	83	792. 50	0. 21	0
45–54	76	735. 00	0. 57	1
55–59	70	327. 50	0. 40	1
60–64	61	280. 00	0. 38	1
65–69	51	235. 00	0. 36	0
70–74	43	180. 00	0. 30	1
75–79	29	130. 00	0. 28	0
80–84	23	72. 50	0. 22	0
85+	6	17. 75	0. 04	0
		Totals	2. 80*	4*

*$X^2 = 0.18$ (d.f. = 1), $P = 0.70$ to 0.50.

TABLE 15.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P1 sisters*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	467	2279. 50	0. 12	0
35–44	445	4055. 75	0. 74	2
45–54	351	2983. 00	1. 33	3
55–59	248	1048. 50	0. 65	1
60–64	177	749. 00	0. 62	2
65–69	119	281. 75	0. 26	0
70–74	75	268. 00	0. 31	2
75–79	40	160. 75	0. 23	1
80–84	27	76. 25	0. 13	0
85+	8	36. 25	0. 08	0
		Totals	4. 47*	11*

*X^2 = 8.21 (d.f. = 1), $P<0.01$.

TABLE 16.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P1 sisters*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	31	155. 00	0. 02	0
35–44	31	260. 50	0. 07	1
45–54	21	151. 50	0. 12	1
55–59	10	43. 75	0. 05	0
60–64	8	31. 50	0. 04	0
65–69	4	17. 25	0. 03	0
70–74	3	13. 25	0. 02	0
75–79	2	7. 00	0. 02	1
80–84	1	1. 00	0. 00	0
		Totals	0. 37*	3*

*X^2 is not shown because of the small observed and expected frequencies.

TABLE 17.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P2 sisters*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	482	2336. 00	0. 12	0
35–44	453	4167. 25	0. 76	0
45–54	376	3255. 00	1. 45	2
55–59	277	1220. 25	0. 76	1
60–64	208	896. 00	0. 74	1
65–69	150	585. 00	0. 54	1
70–74	86	317. 50	0. 36	0
75–79	45	136. 50	0. 19	0
80–84	15	50. 75	0. 09	0
85+	4	14. 00	0. 03	0
		Totals	5. 04*	5*

*X^2 = 0.00 (d.f. = 1), $P>0.99$.

TABLE 18.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P2 sisters*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	42	209. 25	0. 02	0
35–44	41	373. 25	0. 10	0
45–54	33	311. 00	0. 24	0
55–59	26	119. 50	0. 16	1
60–64	20	85. 75	0. 12	0
65–69	14	56. 25	0. 09	0
70–74	8	34. 25	0. 06	0
75–79	5	23. 75	0. 05	0
80–84	4	11. 25	0. 03	0
85+	1	4. 00	0. 01	0
		Totals	0. 88*	1*

*$X^2 = 0.00$ (d.f. = 1), $P>0.99$.

TABLE 19.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P3 sisters*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	307	1498. 25	0. 08	0
35–44	291	2650. 50	0. 49	0
45–54	223	1887. 50	0. 84	1
55–59	147	632. 50	0. 39	0
60–64	105	461. 50	0. 38	1
65–69	78	318. 00	0. 29	0
70–74	44	157. 50	0. 18	0
75–79	19	73. 25	0. 10	0
80–84	8	20. 00	0. 04	1
85+	1	2. 25	0. 00	0
		Totals	2. 79*	3*

*$X^2 = 0.00$ (d.f. = 1), $P>0.99$.

TABLE 20.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P3 sisters*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	43	208. 50	0. 02	0
35–44	40	360. 25	0. 09	1
45–54	30	271. 00	0. 21	1
55–59	20	82. 00	0. 10	0
60–64	13	57. 00	0. 08	0
65–69	9	38. 00	0. 06	0
70–74	5	9. 00	0. 02	0
75–79	1	5. 75	0. 01	0
		Totals	0. 59*	2*

*X^2 is not shown because of the small observed and expected frequencies.

Cousins are the furthest removed genetically from the proband in this group of relatives, and it would be reasonable to expect that the P1 cousins would approach more closely the population value in the number of breast-cancer deaths exhibited than the other 4 groups would. This expectation is realized, since in both ever-married and single P1 cousins, the differences between expected and observed values are not significant. The next 6 tables deal with ever-married and single female cousins in P1, P2, and P3 probands. There are almost 19 expected breast-cancer deaths in ever-married P1 female cousins, and 23 observed (table 21). The difference is not significant: $P = 0.80$. When single P1 cousins are selected as in table 22, the observed value 7 is more than twice that expected (3.13). X^2, however, is not significant at the 5 percent level: $P > 0.05$.

TABLE 21.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P1 female cousins*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	1931	9365.0	0.49	0
35–44	1815	16430.0	3.01	2
45–54	1471	12375.0	5.52	6
55–59	1004	4377.5	2.71	3
60–64	747	3122.5	2.57	3
65–69	502	2035.0	1.88	3
70–74	312	1227.5	1.40	5
75–79	179	607.5	0.85	0
80–84	64	222.5	0.39	0
85+	25	62.5	0.13	1
		Totals	18.95*	23*

*$X^2 = 0.67$ (d.f. = 1), $P > 0.40$.

TABLE 22.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P1 female cousins*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	210	1025.0	0.10	0
35–44	200	1765.0	0.46	2
45–54	153	1310.0	1.01	4
55–59	109	465.0	0.57	0
60–64	77	315.0	0.42	0
65–69	49	197.5	0.30	0
70–74	30	97.5	0.16	1
75–79	9	35.0	0.07	0
80–84	5	12.5	0.04	0
		Totals	3.13*	7*

*$X^2 = 3.68$ (d.f. = 1), $P = 0.10$ to 0.05.

TABLE 23.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P2 female cousins*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	2205	10717. 5	0. 56	0
35–44	2082	18925. 0	3. 47	4
45–54	1703	14420. 0	6. 43	5
55–59	1181	5270. 0	3. 26	8
60–64	927	3880. 0	3. 20	4
65–69	625	2552. 5	2. 36	1
70–74	396	1595. 0	1. 83	2
75–79	242	830. 0	1. 17	1
80–84	94	302. 5	0. 53	2
85+	27	67. 5	0. 14	0
		Totals	22. 95*	27*

*$X^2 = 0.55$ (d.f. = 1), $P = 0.50$ to 0.30.

Ever-married P2 cousins (table 23) show no significant difference between the observed, 27, and expected, 22.95, number of breast cancers. The same is true for the single P2 cousins (5 and 2.86, table 24). Although these differences are not significant, comments have been made that they are, nevertheless, in the direction opposed to the general hypothesis of more frequent breast cancer in P1 relatives than in P2 and P3 relatives. It is inevitable that, if one group of families has more breast cancer than the population average, other groups must have less, though it is not necessary that every *sample* of the population with less should also have less than the average. These sample averages will vary; some will be above expectation, some below, with the average of all of them falling

TABLE 24.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P2 female cousins*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	176	762. 5	0. 08	1
35–44	129	1230. 0	0. 32	1
45–54	117	1070. 0	0. 82	0
55–59	97	427. 5	0. 52	1
60–64	74	305. 0	0. 41	0
65–69	48	195. 0	0. 30	0
70–74	30	117. 5	0. 20	2
75–79	17	62. 5	0. 13	0
80–84	8	22. 5	0. 07	0
85+	1	2. 5	0. 01	0
		Totals	2. 86*	5*

*$X^2 = 0.96$ (d.f. = 1), $P = 0.50$ to 0.30.

a little below the population average as a whole. It would indeed be surprising if *all samples* fell below the expectation.

Tables 25 and 26 show that there is no significant difference between the number of breast-cancer deaths observed and that expected in both ever-married (11 and 16.03) and single (3 and 3.80) P3 cousins. X^2 for the former difference $=1.29$, $P>0.20$, and for the latter $X^2=0.02$, $P>0.80$.

TABLE 25.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in ever-married P3 female cousins*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	1635	7885. 0	0. 41	0
35–44	1519	13570. 0	2. 49	2
45–54	1195	10130. 0	4. 52	2
55–59	831	3662. 5	2. 26	4
60–64	634	2695. 0	2. 22	1
65–69	444	1812. 5	1. 68	1
70–74	281	1072. 5	1. 23	1
75–79	148	530. 0	0. 74	0
80–84	64	212. 5	0. 37	0
85+	21	52. 5	0. 11	0
		Totals	16. 03*	11*

*$X^2 = 1.29$ (d.f. = 1), $P = 0.30$ to 0.20.

TABLE 26.—*Observed and expected frequencies of breast cancer, based on United States mortality data, in single P3 female cousins*

Age group	Number of persons ever present in group	Number of person-years	Expected No. of breast-cancer deaths	Observed No. of breast-cancer deaths
30–34	220	1056. 5	0. 11	0
35–44	203	1800. 0	0. 47	2
45–54	157	1315. 0	1. 01	0
55–59	106	480. 0	0. 59	0
60–64	86	372. 5	0. 50	1
65–69	63	272. 5	0. 41	0
70–74	46	182. 5	0. 31	0
75–79	27	102. 5	0. 22	0
80–84	14	50. 0	0. 15	0
85+	6	15. 0	0. 03	0
		Totals	3. 80*	3*

*$X^2 = 0.02$ (d.f. = 1), $P = 0.90$ to 0.80.

Discussion

The tables show that some factor or factors cause the P1 relatives to reveal significantly more breast-cancer deaths than would be expected if they were experiencing the same risk as the population of the registration area in the United States with which they are compared. These factors

may reside in the environment, or in the genes, or both; or they may be due to a bias in the data. This bias did not exist in the P2 and P3 populations, since the differences between observed and expected values of breast cancer in these samples were not significant. The subject of bias in the P1 data has already been dealt with, and any bias that arose from the method of ascertainment, which is the most important source of bias, was shown not to be sufficient to reduce the difference between observed and expected values to the level of insignificance.

The similarity of environment might be the explanation of the excess breast cancer in P1 relatives. Whatever extrinsic factor was producing breast cancer in the proband might conceivably have been the cause of breast cancer in her sisters, mother, aunts, and grandmothers. Thus, because of cultural patterns handed down from parent to child, the fact that females in a family might tend to have few or no children, or not to nurse the ones they had, would cause an excess of breast cancers in the family, if such factors were important in inducing breast cancer. Similarly, having a large number of children and nursing them might be traditional in the family, and would make for a lower frequency of breast cancer. An objection to this interpretation as wholly or even largely responsible for the excess number of breast cancers is that the same excess exists in paternal as in maternal grandmothers, both sets of grandmothers showing equally increased frequency over that found in the general population.

The paternal and maternal grandmothers were not separated in the table of mortality rates, but in the preparation of proportionate death rates prepared for table 5, this separation into the 2 groups was done. The results were as follows: 7 verified breast cancers among 134 paternal grandmothers—only 1.96 were expected; 10 verified breast cancers among 163 maternal grandmothers—only 2.53 were expected. When the paternal and maternal grandmothers were compared for similarity of age distribution through 13 quinquennial age groups, $X^2 = 13.32$ (d.f. = 12), $P > 0.30$. When compared for decade of death distribution, $X^2 = 1.90$ (d.f. = 4), $P > 0.70$. Mortality rates for breast cancer have changed little over the last few decades, but proportionate death rates have changed; therefore, it became necessary to test for similarity of decade-of-death distribution as well as for age.

Since it was evident that paternal and maternal grandmothers were a homogeneous group, they were then compared directly for the amount of breast cancer which they showed. With a fourfold table for testing similarity for number of breast cancers in these 2 groups, $X^2 = 0.12$ (d.f. = 1), $P > 0.70$.

It would be unusual to find the same environmental situations in the father's and mother's family influencing his sisters and mother to the same extent that it influenced the mother's sisters and mother. On the other hand, from the genetic standpoint, hereditary qualities are passed on equally from paternal and maternal lines to the offspring, except in sex-linked traits. If genetic rather than environmental factors are

mainly responsible, a situation similar to that found in the 2 sets of grandmothers should exist in paternal and maternal aunts. This was true.

P1 aunts were similarly separated into paternal and maternal groups in preparation of age-specific, proportionate death rates. There were 327 paternal aunts with 22 verified breast cancers, and only 6.8 were expected. There were 355 maternal aunts with 21 verified breast cancers, and 7.7 were expected. In order to compare the aunts directly, they were first tested for similarity of age and decade-of-death distribution in the same manner as described for grandmothers; the P values were respectively >0.95 and >0.10. They were then compared directly for similarity of number of breast cancers in a fourfold table, and $X^2 = 0.19$ (d.f. = 1), $P>0.50$. These findings presuppose either an almost identical paternal and maternal environment, which is difficult to accept, or a genetic component in the determination of breast cancer.

It must be remembered that, on the basis of chance distribution of a trait throughout the community, some families will have more than 1 member with the trait. The more common the trait, the more often will multiple cases be found in a given family, without the necessity of invoking either a common environmental or common genetic factor. Cancer of the breast, however, is not so common a characteristic that one expects to find it frequently in 2 sisters, especially in 2 living sisters.

In the P1 families there were 374 living sisters, with an average age of 54. Reference to table 6 shows that the risk of dying with breast cancer, for women between ages 50 to 54, was 62 per 100,000 in 1940. Various workers have estimated that for every woman who dies of breast cancer at any given time there are 3 living who have the disease. About 186 per 100,000 women at the age of 50 to 54 years should have breast cancer. On the basis of proportionate representation, there should be 0.7 women with breast cancer among the 374 living sisters. Actually, there were 10 living sisters, of P1 probands, who also had breast cancer. Again, there is the probability of finding families, with 2 affected, twice as often as there is of finding families with but 1 affected, whether the multiple cases are genetic in origin or due to chance sampling. This leaves 5 families with an additional affected sister, which is still 7 times the expectation. There seems to be no justification for attributing the excess of multiple cases to chance appearance of the condition in more than 1 sister in these families. The same argument applies also to other relatives.

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Deoxyribonucleic Acid Content of HeLa Stock and Newly Established Clone Cultures [1]

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Summary

The deoxyribonucleic acid (DNA) content was determined in individual cells of Feulgen-stained HeLa stock and newly established HeLa clones with a redesigned sensitive cytophotometer. The DNA content varied in cells of the stock. Similar variations in DNA were observed in clones by the time 10 divisions had occurred. Morphologic observations and cytophotometric measurements indicated that the DNA in the particular HeLa cells studied and grown under the conditions described did not reduplicate in a uniformly stable manner in populations which were started from single cells.—**J. Nat. Cancer Inst. 22: 953–960, 1959.**

Chromosome studies of established cell strains from different tissues and from different mammals have shown that alterations in chromosome number or morphology, or both, occur (*1–6*). These alterations may also develop in primary cultures from either normal or malignant tissues (*7–9*). Even clonal populations from an established cell strain have shown not only differences in the chromosomes among cells but also differences in biological and biochemical behavior (*10–12*). Recently Tjio and Puck (*13*) observed that fibroblast-like cell lines, which they developed from tissues of normal human beings and from an opossum, had stable chromosome complements for more than 5 months of cultivation *in vitro*. Their results are the only exceptions to date to the general observations that chromosomes in cells change in morphology and in number after explanting *in vitro*.

The purpose of this study was to determine whether HeLa cells in clones established from single cells would contain equal amounts of deoxyribonucleic acid (DNA). Since cells of this strain multiply rapidly *in vitro*, one might expect to find some cells in the process of synthesizing new DNA. However, if the DNA content was stable, then no cell of a given clone would be expected to have more than twice the lowest value, which would be characteristic of a cell that had recently completed a

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division. Except for 1 clone, these determinations were made when the HeLa clones had reached a population density of 800 to 1,000 cells (before 10 divisions).

Since it was not technically possible to obtain sufficient chromosome counts on small populations to determine the exact chromosome number and morphology, the relative amounts of DNA in individual cells were determined by cytophotometry of Feulgen-stained HeLa nuclei. When carried out under carefully controlled conditions, this method for the determination of DNA has been demonstrated to give reliable, quantitative values in "arbitrary units" (*14, 15*).

Materials and Methods

This laboratory has maintained the HeLa stock line from which the clones were established for the past 4 years in a medium composed of 1 part calf serum and 2 parts medium 199. The method used for the preparation of clones in D-5.0 Carrel flasks has been previously described (*16*). Clone I cells were grown for 3 months. During this time these cells were explanted 10 times at approximately weekly intervals by trypsinization with a 0.05 percent solution of crystalline trypsin (Worthington Biochemical, Freehold, New Jersey) in medium 199. One to two thousand cells of the stock and Clone I cell were explanted in the same way described for the other clones. Clones II to VII were allowed to multiply until there were about 800 to 1,000 cells growing as small compact discs in D-5.0 Carrel flasks for 10 to 14 days. Since some cells were lost during the trypsinization and transfer of cells to slide-cap cultures, it was technically difficult to work with fewer than this number of cells. The cells were then explanted to slide-cap cultures (*17*) for 48 hours, after which time the vaccine vial caps were removed and the cultures rinsed in Hanks' solution and fixed in Carnoy's solution (3 absolute alcohol/1 glacial acetic acid) for 30 minutes. The cells were then hydrated, hydrolyzed with 1 N HCl for 12 minutes at 60° C., and stained by the Feulgen technique (*18*).

The estimation of DNA in individual cell nuclei was done with an instrument modified from the conventional photometers described by Pollister, and others (*14, 19*). Instead of the usual 931-A phototube, a #5819 end-window photo cathode was used. A small lens was mounted in front of the photo cathode so that transmitted light through the mean optical path of a nucleus would be dispersed over a uniformly large area of the photo cathode and would smooth out the well-known point-to-point variations in photo-cathode sensitivity. Plugs from 1 to 4 mm. in diameter were bored in metal plates that were mounted in a 4-place objective holder, which corresponded to plugs of 1 to 4 μ in the subtended image. The plug delineating the light transmitted through the mean optical path of the nucleus (depending on the size of the nucleus) could be chosen by rotating a small knob at the top of the instrument. There was an exact linear relationship between the light transmitted through the plugs of different diameters and the voltmeter response. A high

input resistance vacuum-tube volt meter replaced the conventional galvanometer and a stable, line-operated power supply provided the high voltage necessary for the photomultiplier. The light source, an AH-4 mercury lamp, was used in conjunction with a Baird Multilayer interference filter of narrow band width with a transmission peak at 550 Å. The amount of DNA in a nucleus was computed by multiplying the extinction values by the area ($E \times r^2$) (*20*). All the measurements were made of cells in interphase. In addition to the fine instrumentation and sensitivity of the cytophotometer, the extremely homogeneous distribution of Feulgen dye in the tissue-culture cells added to the accuracy of the measurements.[2]

Results

Before Clones II to VII were established and Feulgen-stained preparations measured, a series of 30 HeLa clones were followed by microscopic observation as they developed from single cells, until there were about 1,000 cells in each clone. Morphologic observations of these clones showed that variations in nuclear size, as well as abnormal mitoses (tripolar and tetrapolar), occurred early in the clones. During the development of the clones, degeneration of a small number of cells was a constant finding in all clones. The detritus of these cells was observed in the closely packed cells of the clones.

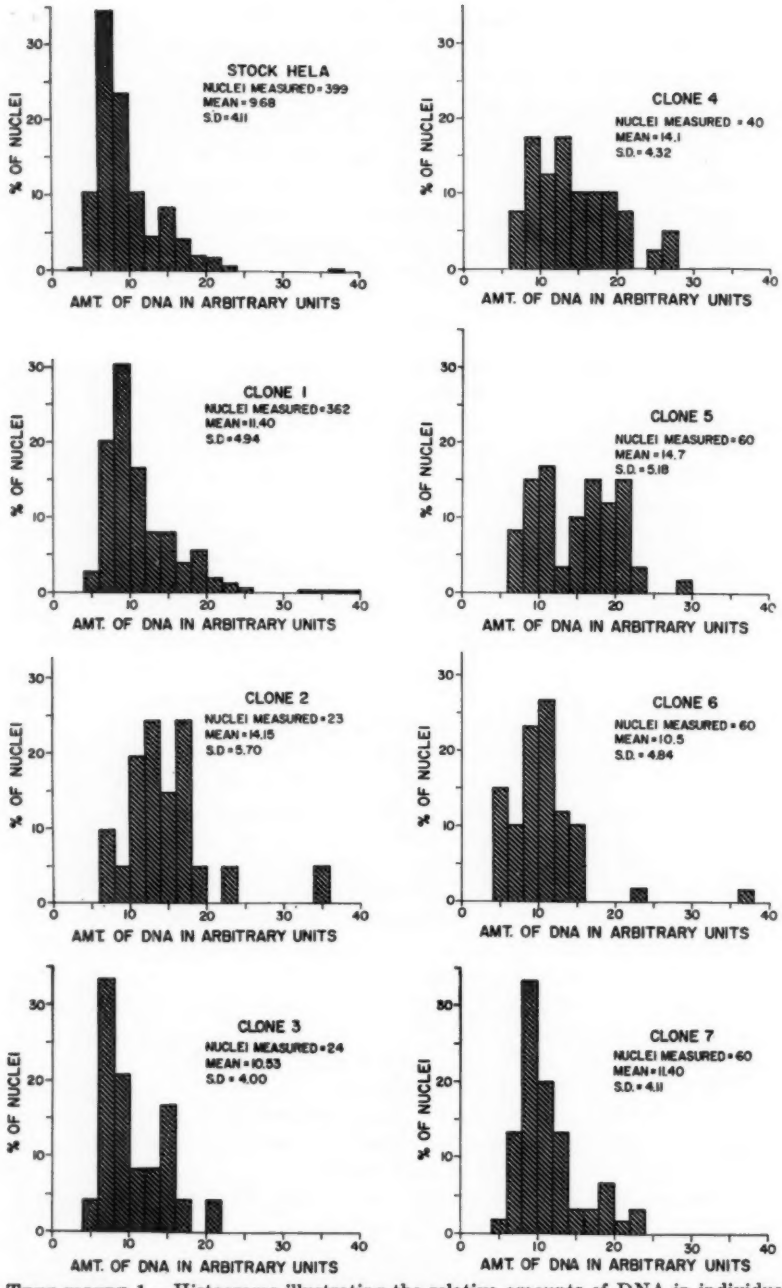
Lymphocytes obtained from the peripheral blood of normal human beings were used for control DNA values in resting cells. All the measurements of the normal blood cells were closely grouped about the mean 4.10 arbitrary units, which in this laboratory is considered to be the normal diploid value in nondividing human cells.

The frequency-distribution histograms of DNA in HeLa cell nuclei of stock and clone cultures are illustrated in text-figure 1. From the very large spread of individual values and the presence of mitotic figures, it was apparent that not only did the HeLa cell nuclei contain different amounts of DNA but also an active synthesis of DNA was occurring. The values obtained in individual cells were too variable and too large (text-fig. 1) to assume that they represented only cells which contained different (resting) amounts of DNA. Determinations in some clones (II, V, and VI) were 5 to 8 times larger than the lowest values in a clone, *i.e.*, 7 arbitrary units compared to 35 arbitrary units in Clone II, and 5 units as compared to 38 units in Clone VI.

The mode or "stemline" value of the stock population of HeLa cells was 9.68 arbitrary units. This value does not indicate that the stem line of this population was composed of hypertetraploid cells, because it is not possible with this method of measurement to correlate the DNA in cells with the chromosome number (*see* Discussion).

The variation in DNA in cells of Clone I that multiplied for 3 months after isolation from a single cell was as large as in the stock HeLa cells.

[2] The cytophotometer (including the power supply) was built by the Department of Instrument Design and Development in this Institute. The electronic circuits were designed by Dr. E. Gordy, Chief of the Department of Instrument Design and Development. A paper describing in detail the instrument and the electronic circuiting is in preparation.



TEXT-FIGURE 1.—Histograms illustrating the relative amounts of DNA in individual HeLa cells as determined by cytophotometry of Feulgen-stained nuclei of stock and clone cultures.

Cells of both the stock and Clone I HeLa lines multiply at the same rate. A statistical analysis of the DNA values in stock and Clone I cells showed that their means were significantly different (9.68 as compared to 11.40) at the 0.001 probability level, indicating that it was very unlikely that the nuclei measured were derived from the same population of cells. One could not distinguish these populations by their mean differences since no studies were carried out to correlate the variability in DNA in these HeLa cell lines at repeated time intervals. It seemed unwise to try to interpret these data as was done by Chu (*5*), who distinguished HeLa clone lines from one another by their chromosomal distributions and by cytophotometric determinations of DNA on small samples of HeLa cells.

The variability in DNA among cells in Clones I to VII was as great as in the stock and Clone I subline (table 1). Although comparatively few cells were measured in some of these clones, the very large individual differences in DNA among cells of these clones indicate that they did not contain equivalent amounts of DNA per cell or amounts approaching double the lowest value obtained. The data clearly indicated that when HeLa sublines were started from a single cell, the DNA did not reduplicate itself in a uniformly stable manner. Instead, variations in DNA appeared among cells of these clones before 10 divisions had occurred.

TABLE 1.—*DNA in HeLa stock and clone cells as determined by cytophotometry of Feulgen-stained nuclei*

Cells	Number of nuclei measured	Mean amount of DNA in arbitrary units	Standard deviation	Standard error of mean
Stock HeLa	399	9. 68	4. 11	0. 20
Clone I	362	11. 40	4. 94	0. 26
Clone II	23	14. 15	5. 70	0. 24
Clone III	24	10. 53	4. 00	0. 82
Clone IV	40	14. 10	4. 32	0. 68
Clone V	60	14. 70	5. 18	0. 67
Clone VI	60	10. 50	4. 84	0. 63
Clone VII	60	11. 40	4. 11	0. 53

Discussion

Studies of the chromosomes of established human epithelial strains indicate that these strains are composed of heteroploid and aneuploid cells (*3*, *4*). The observations of Tjio and Puck (*13*) showed that most of the cells of rapidly growing fibroblast lines (5 to 6 months *in vitro*) originating from normal human tissues contained chromosomes with the normal diploid number and normal morphology. In addition, chromosome studies of HeLa subclone S9, established by these investigators, had an unusually stable chromosome complement (this subclone had been propagated for only a short time). The cells of clone S9 had a more uniform "morphology" than the original clone from which these cells were isolated.

Before the DNA was measured in 7 HeLa clones, microscopic observations of 30 clones showed that cells with variable nuclear sizes and cells

exhibiting tripolar and tetrapolar mitoses developed very soon after cloning. Although we did not make chromosome counts in HeLa stock and clones, it was apparent from our measurements that the clones showed as great a variation in DNA as the stock culture. It is possible that the methods of growing cells *in vitro* developed by Puck and coworkers account for the stable chromosome complements which their cells retain.

Chu's studies of clones isolated and established from HeLa stock cultures by Puck showed variations in chromosome number and morphology within clones and also variations between clones (*5*). These clones were isolated by Puck approximately 2 years before Chu conducted these investigations.

Sanford and coworkers (*10*) found not only a difference in morphology of sublines of mouse cells that had been derived from a single cloned fibroblast but also differences in the ability of these sublines to produce sarcomas in the strain of mice from which the cells originated. Similar findings have been reported by Evans (*21*).

Levan and Biesele (*9*) showed that trypsin may act as a mutagenic agent in newly explanted cultures of mouse tissue and increased the frequency of abnormal mitoses and chromosomal aberrations. Mitotic aberrations, however, appeared with less frequency, but nevertheless were present, in newly explanted mouse tissue cultures even when trypsin was omitted. Similar mitotic aberrations were reported by Kleinfeld (*8*) in trypsin-prepared primary explants of monkey kidney. She found that 16 to 40 percent of dividing cells in culture contained abnormal divisions 6 to 8 days after explanting. Trypsin cannot be the only factor responsible for the chromosome changes in cells since Tjio and Puck's cells were routinely trypsinized. Many tumor lines carried in animals also exhibit such variability and are not exposed to this agent.

In the present studies we made no attempt to correlate directly the DNA content in HeLa cells determined by cytophotometric measurements with absolute quantitative values such as chromosome numbers or DNA in cells determined by biochemical methods. In heteroploid and aneuploid populations multiplying *in vitro* or *in vivo*, one cannot always be certain whether the arbitrary units used by an investigator represent a measurement of DNA in resting cells with given numbers of chromosomes, or represent intermediate values in some cells which are in the process of synthesizing new DNA in preparation for division. Although histograms showing the distribution of chromosomes in cells and those showing distribution of (Feulgen) DNA in cells appear very similar (*1*, *4*, *5*), one must be careful in transposing arbitrary values into exact chromosome numbers.

The differences in DNA-Feulgen content of cells may be due to a number of factors. As Moses [3] has noted, among these could be changes in stoichiometry or absorption spectra of the DNA-Feulgen complex, as well as biological variations caused by DNA synthesis, loss of DNA, or varia-

[3] M. S. Moses, discussant for paper, Changes in Nuclear DNA in Normal and Ascites Tumor Cells, Ann. New York Acad. Sc. 63: 846-848, 1956.

tions in chromosome numbers. Although variation in chromosome numbers in tumor-cell population may be the most probable reason, synthesis of DNA, or any one of the other factors noted, may be involved.

Mellors (*22*, *23*) and Leuchtenberger and coworkers (*24*) have shown that the DNA as determined by ultraviolet or Feulgen cytophotometry varies considerably in tumor cells of some mouse and human tumors. Leuchtenberger assumed that there is a direct correlation between DNA in "resting" tumor-cell populations and chromosome numbers and has even converted arbitrary units obtained from DNA-Feulgen measurements into quantitative amounts of DNA. Until the biological variables discussed previously have been elucidated, one should be cautious in transposing data obtained for DNA by various cytophotometric techniques to data obtained for DNA by biochemical methods or chromosome counts.

Our studies of the DNA in recently explanted clones of HeLa showed the early appearance of cells with apparently different basic amounts of DNA. The data is not intended to indicate that HeLa sublines carried for a long period *in vitro* would not develop stable stem lines; in fact, the DNA measurements of Clone I indicated that this subline was significantly different (in mean DNA content) and this could have been a stable difference if studied over a long period.

Hauschka and Levan (*25*) have shown that clones obtained from Ehrlich and Krebs-2 ascites cells were stable over a 5-year period during serial transfer in mice. These cloned lines had narrower chromosome number ranges than the stock tumor cells from which they were isolated. The clones also exhibited individualities with respect to their virulence when introduced into susceptible hosts.

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Effect of Repeated Brief Stress on Growth of Ehrlich Carcinoma in the Mouse [1, 2]

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Summary

These investigations were directed to the development of stressor systems for the production and maintenance of emotional disturbance in mice, and the application of these stressors to mice, in order to explore their effect on the growth rate of malignancies. In a series of 8 experiments, mice with subcutaneous Ehrlich tumor transplants were subjected to stress either by an avoidance shuttlebox or by confinement. In 5 of the 8 experiments significantly smaller tumors were found in stressed animals as compared to their controls. Two of the remaining 3 experiments yielded nonsignificant results in the same direction, and 1 showed a significant increase in the size of tumors. Exposure to shuttlebox or confinement stress tends to inhibit the growth of Ehrlich tumors in mice, and it is suggested that stress represents a variable to be considered in cancer research. Stress also offers an additional context for the exploration of tumor-host interrelationships.—**J. Nat. Cancer Inst. 22: 961–977, 1959.**

While a number of clinical observations suggests the possibility that various life stresses may constitute one factor in the development of malignancy, this aspect of host-tumor relationships has received little direct experimental testing. Andervont (*1*) and Mühlbock (*2*) investigated the effect of isolated *versus* group rearing on age at onset of "spontaneous" mammary tumors in virgin female mice. Results of these studies show a lower average age of tumor development in isolated animals.

A more direct test of the effect of exposure to stressful situations on host-tumor relationships was reported by Rashkis (*3*), who subjected mice to forced swimming before and after tumor induction by methylcholanthrene or by ascites tumor transplantation. Growth of both types of tumors was inhibited by stress, and survival time of the mice was longer than that of the controls.

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[2] This investigation was supported by U.S. Public Health Service research grant C-2496.
[3] The authors wish to acknowledge the assistance of Normal Q. Brill, M.D., in the initiation of this research.

Reznikoff and Martin (*4*) subjected a mouse strain with a high tumor incidence to a stressful situation involving the administration of brief electric shock at irregular intervals. The time of tumor appearance and development in these mice, which were stressed continuously over a period of several months, was compared with that of control animals. No significant differences were found between stressed and control animals with respect to the incidence of mammary tumors or age at tumor appearance, but a trend toward earlier tumor appearance in stressed animals was apparent.

Recent Russian work (*5*, *6*) along similar lines has yielded results in the same direction. Although descriptions of this work are incomplete, and relevant variables appear to be poorly controlled, the authors report a higher incidence and a more rapid development of various cancers in mice subjected to a conditioning stress than in control animals.

Harvey and Field (*7*) have recently reported extensive studies on the effect of stress on the growth of induced and transplanted tumors in rats. The stressing situation involved a brief shock delivered at regular intervals and preceded by a light of 12 seconds' duration, which served as a conditioned stimulus. Animals maintained in this setting over a 6-month period were subjected to tumor induction by methylcholanthrene injection or by transplantation of Walker carcinoma. No significant differences in tumor incidence, growth rates, and regression rates were found between experimental and control animals. Although not significant, there was a trend toward a higher incidence of tumors in stressed animals. A 50-day stress period prior to tumor transplantation was more effective than concurrent stress in influencing both tumor "takes" and the incidence of regressions.

The studies noted previously are representative of the available literature on the effect of "psychologic stress" on host-tumor relationships. While variations in experimental stressing conditions, choice of host, tumor, and methods of tumor induction, preclude comparisons from study to study, there appears to be little similarity even in the direction of results. Some studies suggest that stress may inhibit tumor growth, some yield equivocal results, while others report facilitation of neoplastic proliferation. The complexity of the problem is in part explained by the multiplicity of variables which affect the host-tumor relationship. The age, sex, nutritional status, and activity of the host, the cancer type and site, and the type of stressor, with its frequency, duration, physiologic and psychologic effect, and period of application in relation to tumor induction, are a few such variables that obviously require consideration. It seems probable that a definitive statement of the effect of stress must await a systematic and exhaustive program of investigation, which will elucidate the basic parameters involved and their interrelationships.

The research described in this report was conceived as the first step in such a program. The experiments were directed primarily toward two goals: 1) the development of reliable stressor systems for the production and maintenance of behaviorally evident emotional disturbance and 2)

the application of these systems, in an attempt to explore their effect on the growth of transplanted tumors.

Methods

Experimental animals and tumor.—Six-week-old female mice, weighing approximately 18.0 gm., from a colony of salmonella-resistant Webster white Swiss mice were used. The Ehrlich mouse carcinoma, carried in stock in the ascites form by intraperitoneal inoculation, was introduced subcutaneously into the flank by the injection of 0.2 cc. of a 10 percent suspension of cells in 0.9 percent saline. A readily measurable solid tumor resulted that killed the host in approximately 45 days—an interval well adapted to the design of subsequent experiments.

At 4-day intervals mice were weighed, and the tumor size was measured with calipers along the largest diameter and averaged with a second measurement taken at right angles to the first. Subsequent data tables are based on these linear measurements. Tumor areas are not computed. A rank order correlation of 0.87 was obtained between tumor measurements taken by independent observers.

All mice were autopsied, the tumor and vital organs weighed, and tissue sections of the heart, lung, kidney, liver, spleen, and primary tumor prepared for microscopic study.

Stressor Systems

Criteria for selection.—The selection of a stressor system was determined by several considerations: 1) The desire for a situation that would produce *sustained* fear. 2) Applicability either continuously or repeatedly over considerable periods, to achieve the first objective. 3) The elimination, or at least minimization, of physical stressors involving trauma which might influence the tumor growth rate. 4) The use of a stressor permitting maintenance of normal nutritional status in the experimental animals. This provision eliminated a number of highly effective approach-avoidance situations such as the interposition of a charged grid between the animal and food or water so that the satisfaction of hunger or thirst required the animal to accept a painful shock. While such situations often result in severe behavioral disturbances, they tend also to impair nutrition, a factor known to influence grossly tumor growth. 5) Applicability to a large number of animals required for exploratory studies.

Shuttlebox stressor.—The shuttlebox stressing procedure finally selected was an avoidance-learning situation, which required the animals to jump a barrier at a light-buzzer signal to avoid a 30-volt shock at frequent intervals.

The shuttlebox paradigm satisfies requirements 1, 2, 4, and 5 of a satisfactory stress situation. Fear is manifested by crouching, tremor, vigorous escape efforts, urination, and defecation. At the same time, the animals, because of avoidance responses, are subjected to shock at only

a small percentage of stimulus presentations, partially fulfilling requirement 3.

Shuttlebox equipment.—One hundred stress compartments were available, approximately 10 × 4 inches and 5 inches deep, with grid flooring, each housing 1 animal. A 1-inch high plastic barrier divided each compartment into two 4 × 5-inch sections.

All experiments utilized the following sequence of stimuli which were electrically operated: A 60-cycle, 30-volt charge was applied to one side of the cages for 5 minutes. Simultaneously, when the charge was removed, a bright light and a loud buzzer in the vicinity of the cages were turned on. These signals lasted for 5 seconds and constituted the cue to jump the barrier in order to avoid the shock. At the "off" for light and buzzer, voltage was reapplied, but to the opposite side of the cage previously occupied by the mouse. This sequence of events was repeated daily for 6 hours, with animals jumping the barrier at the light-buzzer signal every 5 minutes to avoid the shock.

Confinement stressor.—The avoidance-learning stress procedure just described leaves unanswered, to some extent, the question of a possible direct effect of shock on physiologic mechanisms related to tumor growth. Such an effect seems unlikely in view of the low amperage levels (0.75 ma.) involved, and the infrequent shock after avoidance is learned. However, a confinement and immobilization stressing procedure that did not involve the use of shock was added to circumvent this difficulty, and to provide a second stressor, which differed from the first in its effect on the animals.

Experimental animals were loosely wrapped in 4 × 5-inch sheets of fine mesh, copper screen, which produced effective immobilization but at the same time permitted some movement of the limbs and did not restrict respiration. The sheets were folded to 2 × 4-inch dimensions and stapled with one 2-inch edge left open for inserting and removing the animal.

Feeding devices.—Feeder cups containing finely ground Purina mouse pellets were mounted in the wire-cloth top of a larger container which served to collect spillage. A heavy iron washer inserted on top of the food within the inner cup further reduced scattering. Almost all spillage was caught by the outer container, added to the amount remaining in the cup, and weighed after feeding periods. In the one controlled-intake study, after several days of adaptation to individual cages and feeders, animals were paired by weight, and assigned to either stress or control groups. Food offered the experimental animal in each pair was then adjusted to the control-animal-intake of the previous day. This procedure served to match fairly closely the food intake in each pair. After adaptation, food intake, so controlled, did not differ appreciably from the amount of *ad libitum* feeding.

Experimental Procedures

Shuttlebox experiments.—Mice were sorted so as to equalize litter representation and mean weight in experimental and control groups. Each animal was marked for identification, received an injection of tumor, and

was individually housed, with food and water available *ad libitum*. Beginning with the day of injection, the animals were transferred from home cages to shuttleboxes and subjected to the stressing procedure for 6 hours. Control animals were placed in identical cages and received light and buzzer stimulation, but no current. Animals were observed several times per day for avoidance-responses and signs of disturbed behavior. At the end of the 6-hour experimental period all animals were replaced in home cages. This procedure was continued for 15 days following tumor injection or, in the one experiment with preinjection stress, 3 days after injection. Thereafter, animals were killed, or they remained in home cages until moribund.

Confinement experiments.—In one experiment (#9a) animals were housed 5 per cage, while individual housing was utilized in the other (#11). Animals were left in home cages for several days to adapt to new surroundings prior to onset of the stressing procedure.

On experimental day 1, all mice were given the subcutaneous flank injection of Ehrlich's tumor. Experimental animals were then placed in individual screen envelopes for 6 hours daily. During this period food and water were withdrawn from controls to equalize the factor of accessibility. After 15 days, animals were killed in one experiment (#9a) and, in the other (#11), were maintained in home cages, with food and water available *ad libitum* until moribund. In the latter case the remaining animals were killed at day 52.

Table 1 summarizes the type of experiment, stressor applied, number of animals used, and length of follow-up. In 7 of the 8 shuttlebox experiments the 15-day stressing procedure was commenced on the day of tumor injection. In 1 additional study (#2), the experimental group was stressed for 12 days prior to tumor inoculation and for 3 days subsequent to inoculation.

Results

Results are considered in terms of average linear tumor-size measurements taken throughout the course of the experiment, tumor weights (when animals were sacrificed at stress termination), and the presence or absence of metastases. Extent of metastases was not considered since this was small and usually confined to a few organs.

Shuttlebox Stressor Experiments

Experiment 1.—The tumor growth findings are summarized in table 2. During the 2d week of the 15-day stress period, the size of tumors in the stressed group slightly exceeded that in controls. By the 19th day, and thereafter, the average tumor size in experimental animals was exceeded by the controls. One control mouse died early in the experiment before tumor measurements were made. A second control mouse died on day 25 with a 5.5-gm. tumor and a total body weight of 25.6 gm. Experimental mouse deaths occurred on days 40 and 41. The difference in mean tumor size, based on 8 control and 10 experimental mice, is significant on the

TABLE 1.—*Outline of experiments performed*

Expt. No.	Number of mice		Type of stressor	Food intake	Tumor used	Length of follow-up
	Experimental	Control				
1	10	10	Shuttlebox	Not measured	Ehrlich	Survival
2*	20	20	"	"	"	"
4	20	19	"	"	"	"
5	20	20	"	"	"	"
7	20	20	"	Measured	"	Killed, day 16
8	20	20	"	Paired	"	" day 16
9	20	20	"	Measured	None	Survival
9a†	33	30	Confinement	Not measured	Ehrlich	Killed, day 16
10‡	30	30	Shuttlebox	Measured	None	Survival
11	30	30	Confinement	Not measured	Ehrlich	Killed, day 30

*Stressed 12 days before inoculation, 3 days after.
†Housed 5 to a home cage.
‡Also 30 home cage controls.

27th, 30th, and 34th days. Beyond this point the variability within each group became so large as to obscure differences between groups. Body weight averages for the two groups differed by less than a gram up to the 39th day when tumors were large enough to account for a significant proportion of body weight. Thereafter body weight differences became progressively larger. Although directional association was not consistent throughout the experiment, smaller average tumor size tended to be associated with smaller average body weight. It is interesting, however, that at the point of maximum tumor-size difference (day 34) the body weight averages differed by only 0.4 gm. The last control mouse died on day 61, whereas the last 2 surviving experimental mice were killed on day 77. One of these had experienced a complete tumor regression. Five animals in each group manifested metastases.

TABLE 2.—*Experiment 1: Mean total body weight and tumor size*

Experimental (*day*)	Mean body weight (*gm.*)		Mean tumor size (*cm.*)		*P* level of difference in tumor size*
	Experimental	Control	Experimental	Control	
4	19.2 $N = 10$	18.9 $N = 9$	0.74	1.07	—
8 Period of stress	21.0	21.2	1.38	1.26	0.10
11	22.1	22.6	1.56	1.48	0.12
15	23.4	22.7	1.90	1.82	0.20
19	21.7	22.0	1.92	2.01	0.30
24	25.3	25.9	2.01	2.39	0.20
27	26.3	26.6 $N = 8$	1.92	2.32	0.02†
30	27.0	27.5	2.14	2.59	0.02†
34	29.8	30.2	2.38	2.86	0.015†
39	30.3 $N = 9$	34.7	2.56	3.34	0.15
43	31.0 $N = 8$	37.3	2.58	3.55	0.15

*Obtained from Mood test.
†Significant difference.

Experiment 2.—As previously noted, this experiment differed from others of the series in that stress was applied for 12 days before tumor inoculation, and continued for the 3 days following. This procedure provided for the termination of stress just as tumors became palpable.

Table 3 shows that the differences in tumor growth rates of the surviving mice were in the same direction as those found in the first experiment, with average tumor size in the control group exceeding that of experimental animals, from the 4th day following tumor appearance. In contrast to experiment 1, tumor size variability within groups was very large, and even the rather sizable mean differences occurring in the latter part of the experiment were not statistically significant. Metastases were observed in 2 experimental and 3 control mice. Mean body weights for the two groups did not differ significantly.

TABLE 3.—*Experiment 2: Mean total body weight and tumor size*

Experimental (*day*)		Mean body weight (*gm.*)		Mean tumor size (*cm.*)		*P* level of difference in tumor size
		Experimental	Control	Experimental	Control	
1	Period of stress	$N = 20$	$N = 20$			
9		20. 0 $N = 19$	20. 2 $N = 19$			
12*			$N = 18$			
13		20. 4	20. 5			
17		21. 8	21. 8	0. 71	0. 72	Wilcoxen unpaired replicates test $P > 0.05$ in all comparisons
21		22. 2	22. 6 $N = 17$	1. 32	1. 38	
25		21. 8 $N = 18$	23. 5	1. 66	1. 82	
29		24. 3 $N = 17$	23. 8	1. 69	1. 82	
33		25. 7	25. 5	1. 90	2. 01	
37		27. 0	26. 2	1. 86	2. 18	
41		28. 5	28. 2	1. 92	2. 28	
45		29. 3	29. 0	2. 18	2. 59	
49		28. 7 $N = 14$	30. 8	1. 88	2. 72	

*Day of tumor inoculation.

Experiment 4.—As is evident in table 4, significant differences are found between groups on the 6th and again on the 14th day, continuing through the 26th day of the experiment. Again the direction of the difference suggests an inhibition of tumor growth in the stressed group. Since no deaths occurred between days 6 and 30, the differences in this case are not complicated by a declining N value.

The close correlation of body weight and tumor size averages is clear in this experiment, though at one point (day 14), when mean body weights were equal, a very significant difference occurred in mean tumor size.

Tumors metastasized in 8 of 17 stressed animals or 47 percent, as against 15 of 18 control animals or 83 percent. This difference is significant ($P = 0.02$) by chi-square test.

Experiment 5.—Significantly smaller tumors were found in stressed animals on the 11th, 15th, and 24th days (table 5). All mice survived during these observations. The first death occurred in the control group at day 31.

TABLE 4.—*Experiment 4: Mean total body weight and tumor size*

Experimental (*day*)		Mean body weight (*gm.*)		Mean tumor size (*cm.*)		*P* level of difference in tumor size *
		Experimental	Control	Experimental	Control	
1	Period of stress	*N*=20	*N*=19			
3		19.5 *N*=19	20.0 *N*=19	0	0	—
6		21.2 *N*=17	21. 6	0. 38	0. 43	0. 045†
11		21. 8	21. 0	0. 83	1. 01	0. 15
14		22. 7	22. 7	0. 99	1. 28	0. 003‡
18		24. 5	25. 8	1. 27	1. 60	0. 003‡
21		25. 9	26. 8	1. 52	1. 87	0. 02†
26		27. 3	30. 6	1. 87	2. 37	0. 016†
30		31. 1	33. 6	2. 23	2. 58	0. 30
33		35. 2 *N*=16	34. 4 *N*=14	2. 49	2. 61	0. 80

*Obtained from Mood test.
†Significant difference.
‡Very significant difference.

Again the association between body weight and tumor size appears to be close. To test this possibility a series of X^2 tests was applied to four-fold tables constructed by dichotomizing the mice on these variables. The cutting score used was the median of all animals for the weight and tumor size variables. The degree of association was found to increase with successive measurements. No significant association occurred through the 11th experimental day, but the 15th and subsequent days, through 24, do show significant association at about the 0.01 level.

Seven of 18 stressed animals or 39 percent, and 14 of 17 control animals or 82 percent had metastases. The difference is significant at the 0.001 level.

Experiment 7.—The prime objective of this experiment was to see whether food intake, which was measured but not controlled, would differ

TABLE 5.—*Experiment 5: Mean total body weight and tumor size*

Experimental (*day*)		Mean body weight (*gm.*)		Mean tumor size (*cm.*)		*P* level of difference in tumor size*
		Experimental	Control	Experimental	Control	
1	Period of stress	*N*=20	*N*=20			
4		18. 9 *N*=20	19. 2 *N*=20	0	0	—
8		19. 6	19. 4	0. 52	0. 72	0. 11
11		20. 6	21. 1	0. 77	0. 91	0. 008†
15		21. 7	22. 8	1. 09	1. 27	0. 045‡
19		23. 6	24. 7	1. 38	1. 56	0. 15
24		25. 5	27. 4	1. 60	1. 77	0. 05‡
27		27. 2	29. 1	1. 81	1. 95	0. 70
30		29. 6	31. 2	2. 06	2. 20	0. 70
34		31. 8	33. 2 *N*=19	2. 30	2. 44	0. 40

*For chi square based on Mood test.
†Very significant difference.
‡Significant difference.

between stressed and control groups, and what, if any, relations could be discovered between intake, body weight, stress, and tumor growth. The standard post-inoculation, 15-day shuttlebox stress was used.

Animals were housed individually, with a feeder installed in each home cage on the 6th day, when feeders became available. All mice were killed on day 16, following termination of the stress period.

Mean body weight, food intake, and tumor size data are provided in table 6. Mean body weights do not differ by more than 0.6 gm. on any day, and the differences vary during the experiment.

Similarly, no differences in tumor growth which exceeded the 0.15 level were apparent in this experiment. Although mean values were very slightly higher in control animals, the difference is negligible. No metastases were found in either group. At autopsy, mean tumor weights were 1.71 and 1.68 gm. in the experimental and control groups, respectively, confirming the accuracy of the tumor measurement.

TABLE 6.—*Experiment 7: Mean food intake, total body weight, and tumor size*

Experimental (*day*)	Mean food intake (*gm.*)		Mean body weight (*gm.*)		Mean tumor size (*cm.*)	
	Experimental	Control	Experimental	Control	Experimental	Control
2	$N = 20$	$N = 20$	19. 3	19. 7		
4	$N = 19$		19. 6	19. 9		
6			20. 0	20. 2	0. 73	0. 83
7	1. 49	1. 74	19. 5	20. 0		
8	2. 47	2. 75	19. 0	19. 2	0. 91	1. 01
9	3. 04	3. 12	19. 7	20. 1		
10	3. 39	3. 10	20. 3	20. 3		
11	3. 00 (P=.06)*	2. 71	21. 2	21. 3	1. 08	1. 13
12	3. 41 (P=.07)	2. 91	21. 7	22. 1		
13	3. 24 (P=.05)	2. 86	21. 8	21. 2	1. 16	1. 19
14	3. 18 (P=.02)	2. 79	21. 7	21. 8		
15	3. 19	3. 01	22. 2	22. 0	1. 27	1. 33

*Significant differences indicated by numbers in parentheses.

Mean food intake did show minor differences. Intake was probably initially affected by addition of the feeders. This was indicated by body weight loss in both groups and by intake, which almost doubled by the 3d day after addition of the feeders when adjustment to them was achieved. Although intake was initially greater in the control group, experimental animals were eating more by day 10, and the intake difference was very near or at statistical significance for days 11 through 14. However, the sum of all measured mean intakes during the course of the experiment from day 7 to day 15 differed by only 1.42 gm. per mouse. As in previous experiments the similar mean weight values in the two groups are accompanied by similar mean tumor-size values.

Experiment 8.—The effect of controlled food intake is demonstrated in this experiment. The mean total-body weight values (table 7) show that even when intake was matched by pairs, with the stressed mouse

being offered only the amount of food consumed by his paired control on the previous day, stressed animals gained weight more rapidly than their controls. The difference in mean body weight became significant on the 8th day and, with one exception (day 14), remained so through the 15th day of stress.

Similarly, significant differences were found in linear tumor sizes in a direction opposite to that of previous experiments. At autopsy on day 16, mean experimental tumor weights were 2.21 gm. and controls were 1.97 gm. Metastases were not observed.

Experiment 9.—This experiment reduplicated the conditions of experiment 7, measuring food intake in a free-feeding situation, but no tumor was used. The mice remained in home cages after the 15-day stress period until killed at day 52. Food intake and body weight were measured daily throughout the experiment.

TABLE 7.—*Experiment 8: Mean food intake, total body weight, and tumor size*

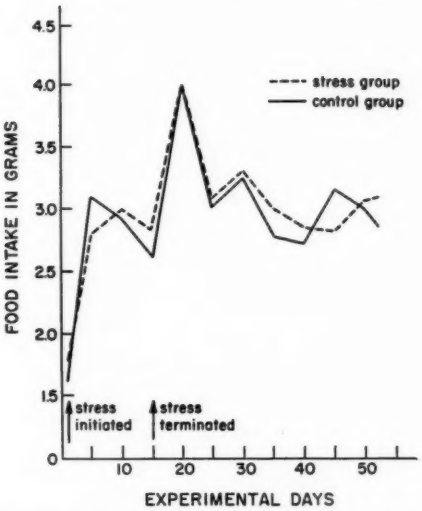
Experimental (*day*)	Mean food intake (*gm.*)		Mean body weight (*gm.*)		Mean tumor size (*cm.*)	
	Experimental	Control	Experimental	Control	Experimental	Control
1	1.08 $N = 20$	1.66 $N = 20$	18. 6	18. 6		
2	2. 33	2. 33	17. 8	18. 1		
4	2. 87	2. 87	18. 5	17. 3		
6	2. 47	2. 47	18. 9	17. 7		
8	2. 44	2. 44	19. 6	17. 8	0.92 (P=.02)*	0. 77
10	2. 69	2. 69	20. 3	17. 9	1.07 (P=.05)	1. 01
12	2.91 $N = 19$	2. 91	20. 8	20. 6	1.24 (P=.01)	1. 08
14	3. 21	3. 21	21. 3	18. 7		
15	3. 18	3. 24	21. 9	19. 5	1.53 (P=.05)	1. 36

*Significant differences indicated by numbers in parentheses.

No consistent or significant differences of intake appeared between stressed and control groups (text-fig. 1). There was a striking increase in the intake of both groups following the termination of stress when food and water first became available for 24 hours per day. However, intake rapidly returned to the previous level within a few days, suggesting that the 18-hour feeding time provided during the stress period was adequate.

In contrast to previous experiments, weight gain was about equal in both groups throughout the 15-day stress period (text-fig. 2). Thereafter the mean body weight of stressed animals increased at a faster rate than the controls, with the difference reaching significance on day 31 only. Beyond this point, mean stressed group weight leveled off so that mean weights were equal by day 52.

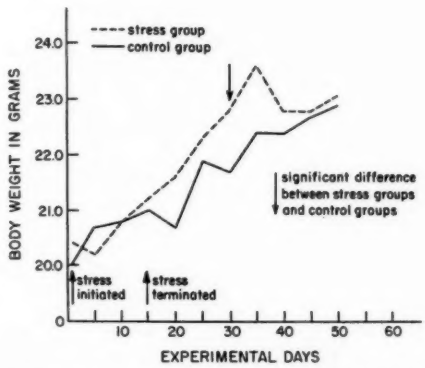
Experiment 10.—The procedures of experiment 9 were duplicated in this experiment. In order to explore the possible effect of daily handling and transfer to stress cages, a third home-cage control group was added. These latter animals were maintained constantly in individual home cages from which food and water were withdrawn 6 hours per day during periods



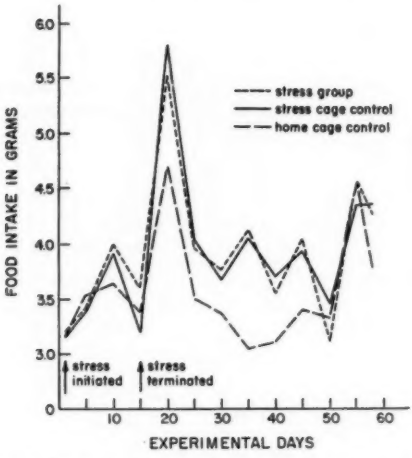
TEXT-FIGURE 1.—Food intake of stressed and control groups.

when the other two groups were transferred to the stressing equipment. Animals were killed at day 58.

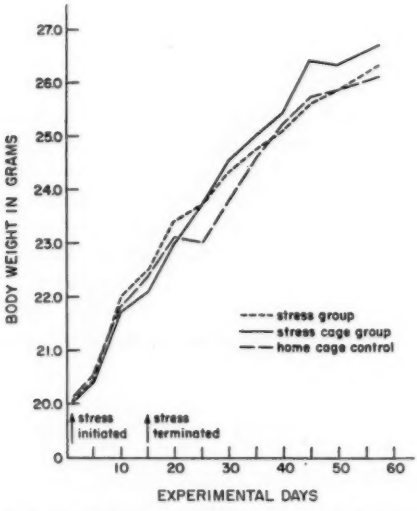
Text-figures 3 and 4 summarize the food intake and body weight data. Although great daily variation occurred in food intake, the three groups tended to vary in the same direction. Only two significant differences were found (days 3 and 5) between stressed and stress-cage control groups, and these were in the opposite direction. The home-cage control group tended to eat less than the other groups, and intake differences between these animals and both stress and stress-cage control mice were frequently significant. Mean body weight curves show little variation with no significant weight differences appearing between groups throughout the experiment.



TEXT-FIGURE 2.—Body weight of stressed and control groups.



TEXT-FIGURE 3.—Food intake in stress, stress-cage control, and home-cage control groups.



TEXT-FIGURE 4.—Body weight changes of stress, stress-cage control, and home-cage control groups.

Confinement Stressor Experiments

Experiment 9a.—The 4 tumor measurements (table 8) made during the stress period yielded higher average tumor sizes for control animals in each instance, but the data were not regarded as reliable because of difficulties in tumor measurements resulting from a more caudal tumor location. Accordingly, animals were killed at the end of the 15th day. Average tumor weights were 1.15 gm. for experimental animals and 2.52

TABLE 8.—*Experiment 9a: Mean total body weight and tumor size*

Experimental (*day*)	Mean body weight (*gm.*)		Mean tumor size (*cm.*)	
	Experimental	Control	Experimental	Control
1	20. 3 $N = 33$	20. 5 $N = 30$		
3	18. 4	21. 3		
5	18. 6	21. 9		
7	19. 0	21. 8		
9	18. 6	21. 9	0. 58	0. 70
11	19. 3	22. 1	0. 86	1. 15
13	19. 6	23. 2	1. 18	1. 41
			Tumor weight (*gm.*)	
When killed	19. 8	23. 6	1. 15	2. 52

gm. for the controls. A Mood test of this difference yields a chi-square of 4.52, which is significant beyond the 0.05 level.

As previous results had led us to expect, no indication of metastases appeared on microscopic examination of tissue sections. This finding suggests that the Ehrlich tumor transplanted subcutaneously, at the locus we used, metastasizes relatively late in the course of tumor development.

In this run, the difference in mean total body weight favoring the control group was evident after a single day of stress by confinement and became increasingly large with successive measurements. Carcass weights declined 1.7 gm. in the experimental group and 0.7 gm. in the controls from day 1 until killed on day 16.

Mean adrenal weights were slightly higher in stressed animals than in controls and spleen weights considerably lower. The latter difference was significant at a P level beyond 0.01.

Experiment 11.—Smaller differences in average tumor size and less variability within groups were found in this experiment in which the animals lived for a longer term (table 9). Average tumor size in stressed animals was significantly smaller than that in controls, at only one point

TABLE 9.—*Experiment 11: Mean total body weight and tumor size*

Experimental (*day*)	Mean body weight (*gm.*)		Mean tumor size (*cm.*)		P level of difference in tumor size*
	Experimental	Control	Experimental	Control	
1 (Period of stress)	18. 8 $N = 30$	18. 7 $N = 30$			
5	18. 4	19. 5			
9	19. 5	20. 4	0. 41	0. 55	
13	20. 1	21. 2	0. 98	1. 01	
15	20. 5	21. 4	1. 14	1. 30	0. 10
17	21. 6	22. 5 $N = 29$	1. 22	1. 34	0. 06
19	22. 5	23. 1	1. 34	1. 51	0. 02†
21	24. 1	23. 8	1. 52	1. 50	
24	24. 6	24. 5	1. 61	1. 66	
28	25. 2 $N = 29$	25. 4	2. 01	1. 99	
30	26. 4	26. 1	2. 04	2. 05	

*For chi square based on Mood test.
†Significant difference.

TABLE 10.—*Summary of results*

Expt. No.	Food intake	Day of termination	Maximum significant difference in tumor size		Day of maximum significant difference	Tumor weights		Metastases	
			Experimental (*cm.*)	Control (*cm.*)		Experimental (*gm.*)	Control (*gm.*)	Experimental (*percent*)	Control (*percent*)
1	—	39	2. 38	2. 86	34	—	—	50	50
2	—	49	None	None	—	—	—	12	18
4	—	33	0. 99	1. 28	14	—	—	47	83
			1. 27	1. 60	18				
5	—	61	0. 77	0. 91	11	—	—	39	82
7	No difference	15	None	None	—	1. 71	1. 68	None	None
8	Paired	15	1. 24	1. 08	12	2. 21	1. 97	None	None
9	No difference	52	—	—		—	—	—	—
9a	—	15	1. 18	1. 41	13	1. 15	2. 52	None	None
10	No difference	58	—	—		—	—	—	—
11	—	30	1. 34	1. 50	19	5. 72	5. 47	30	27

during the run, and on 2 other days the mean of the stressed group was very slightly higher.

Incidence of metastases was almost equal in the two groups with 9 of 30 experimental animals or 30 percent and 8 of 30 controls or 27 percent with metastases.

At autopsy on day 30, tumor measurements were again confirmed by mean tumor weights of 5.72 gm. for the experimental group and 5.47 gm. for the controls.

Summary of Results

As summarized in table 10, 8 of the 10 experiments reported were designed to test the effect of applied stressors on the growth of malignancies. Significantly smaller tumors were found in stressed animals, as compared to their controls, at some point during 5 (# 1, 4, 5, 9a, and 11) of the 8 experiments. In 2 of the remaining 3 experiments (# 2 and 7) nonsignificant differences in the same direction were found. One experiment (# 8) showed a significant increase in the size of tumors of stressed animals as compared to controls.

In general, the results of these experiments suggest that the growth of subcutaneous transplants of the Ehrlich tumor in mice may be inhibited in animals subjected to either of two differing stressful situations.

Discussion

In these studies the inhibition of tumor growth is, of course, modest as compared with the effect obtainable from administration of chemical agents, and somewhat less predictable. These considerations suggest the operation of relatively subtle, indirect, and, perhaps, nonspecific mediating mechanisms or causal factors.

In considering possible causal factors it is of some interest to compare the two stressor systems, which have yielded similar results but differ in several of their effects on the organism.

For example, some studies have shown physical activity to be a factor in the host-tumor relationship. The stressors we employed differ greatly with respect to this variable. In the shuttlebox situation, activity on the part of the stressed animals is enforced, at least to the extent of frequent barrier jumping. In an effort to equalize stress and control group activity to some degree, food and water were removed during stress periods to stimulate foraging, and outside light-dark cycles were reversed in the laboratory so that experimental periods occurred in darkness. Despite these measures, experimental animals were sometimes observed to be more active than controls.

In the confinement experiments, gross physical activity is obviously curtailed. Some struggling occurred after the mice were inserted in the screen envelope but, in general, confined animals lay quietly during the stress period. It is reasonable to assume that the stressors influence physical activity in opposite directions. It seems unlikely that activity represented a crucial, independent variable in these experiments.

In the shuttlebox situation a second point of difference is the use of electric shock only. Conceivably shock might directly affect tumor growth. Repeated correlations between percentage of successful avoidance of shock and tumor size or weight in the shuttlebox experiments ranged about zero. In addition, the similarity of results in the confinement and shuttlebox situations is an argument against shock *per se* as an important independent variable.

At least two other possibilities remain. The first concerns the effect of food intake and body weight on tumor growth. The relationship between tumor size and host nutrition is well known. Our data are quite consistent in indicating a correlation between tumor size and total body weight of the host with smaller tumor size associated with smaller body weight. At the same time, our data do not provide a clear-cut indication of the direction of cause and effect in this relationship, nor can we be sure in all experiments that the difference in total body weights between stressed and control animals represents a real difference in carcass weight. In 4 experiments in which animals were killed at the termination of stress, it was possible to obtain carcass weights. Averages ranged from 17.5 to 21 gm. In 2 of these experiments (#7 and 11), mean stress and control group carcass weights did not differ appreciably at the time the animals were killed. In the others (#8 and 9a), mean carcass weights differed by about 2 gm. or approximately 11 percent of average carcass weight. In both cases the larger tumor-size group was also the heavier carcass-weight group.

Assuming a real body weight difference in those instances where a significant difference in tumor size was found, the question is raised whether the stressing procedure merely influenced intake sufficiently to restrict the supply of essential nutritive materials resulting in reduced body weight and impaired tumor growth vigor. The 2 experiments exploring this question with tumor-bearing animals are not sufficient to provide a clear-cut answer. In one case, food intake of the stressed group exceeded that of the controls, yet tumor size and total body weight were similar. In the other experiment, when stressed animal intake was matched to the intake of the controls, stressed animals gained more weight and had larger tumors. However, results of the two studies exploring the effect of stress *per se* on intake and body weight in non-tumor-bearing animals are consistent and unequivocal. They can be summarized with the statement that shuttlebox stress in the absence of tumor exerts little or no consistent influence on food intake or body weight. On the basis of these results it seems likely that the stressor-food intake-body weight-tumor size interaction is a highly complex one. Whereas body weight differences and tumor size differences are consistently correlated in our data, and the former may represent an antecedent influence upon tumor growth rates, these body weight differences are not clearly related to stress or food intake.

Finally, to be considered, is the effect of the stressor on the host. In the experiments reported here, the animal is placed in a noxious environ-

ment from which he cannot escape and, in the case of the shuttlebox, this poses the continuing problem of precise reaction to a signal in order to avoid ensuing painful consequences. Continued exposure to these situations results in observable behavioral disturbance manifest in crouching, immobility, violent escape efforts, tremors, vocalization, and defecation. Presumably these observed disturbances are accompanied by alterations of homeostatic functions mediated through nervous and humoral mechanisms. Recent exploration (*8*) of responses in non-tumor-bearing animals subjected to both of our stressors reveals that prolonged 12- to 28-day exposure for 6 hours per day results in significant and predictable physiologic alterations. In brief, these are adrenal hypertrophy, shrinkage of spleen and thymus, and drop in total white blood cell counts. It is premature to appraise these evidences of altered adrenal functions in terms of specific hormone outputs. These changes are reversible and organ weights return to normal levels following the cessation of stress.

We can, of course, only speculate at this point on the effect this demonstrated alteration of adrenal function may have on the tumor-host relationship in our studies. Drastic alterations of adrenal hormone levels, obtained through ACTH or adrenal steroid medication, and adrenalectomy have both influenced the growth rate of some neoplasms. Possibly the relatively small suppression of tumor growth effected by our stressors is a manifestation of more physiologic alterations of adrenal function. It is also conceivable that other, as yet unmeasured, homeostatic mechanisms unrelated to adrenal function may have been influenced by the psychologic stress employed.

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Irradiation of Mouse Skin With Single Doses of Ultraviolet Light [1,2]

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Summary

Changes in the ears of albino mice, following single exposures to polychromatic ultraviolet radiation, have been studied with a method that insures uniformity of incident dosage. Gross and histological changes and quantitative evaluation of hyperplasia indicate a pattern of inflammatory lesion followed by healing processes, but with certain special characteristics that may be associated with the restriction of the primary action of the radiation to a very thin layer. Relationships of these studies to the induction of cancer by repeated doses of ultraviolet radiation are discussed.—**J. Nat. Cancer Inst. 22: 979–993, 1959.**

Although induction of skin cancer in mice and rats by repeated doses of ultraviolet light has been the subject of a number of studies (*1*), the events which precede the appearance of cancer have not been investigated systematically. Grady, Blum, and Kirby-Smith made preliminary studies in connection with their investigations of carcinogenesis, but the conditions of irradiation rendered interpretation uncertain, and the matter was not pursued. The present paper describes a method for irradiation of the skin of the mouse ear, which permits reproducible dosage, and reports some of the changes that follow single exposures.

Experimental Method

The principal new features of the method are embodied in an apparatus, which is described in figure 1, to hold the mouse during irradiation. The animal, anesthetized with sodium pentobarbital (Nembutal®), was placed on the metal platform, T, with its head between 2 adjustable metal

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plates, P, with surfaces at 45° to the horizontal; upon these the ears of the animal were flattened during irradiation. The metal surface was covered with a sheet of soft paper, and the ear held against this by a thin sheet of fused quartz, L. Microscope slides made from fused quartz with high transmission in the ultraviolet were used. Since these had the same dimensions as the metal plates (1 × 3 inches) beneath them, the slide and ear could be held in place by the tension of a rubber band, as indicated in figure 1. The ears were exposed to a beam of ultraviolet radiation directed normally to the surface of the quartz slide, part of the ear being shielded from the radiation by a piece of aluminum foil or cellophane, opaque to the effective wavelengths shorter than 0.32 μ, which was inserted between the slide and the ear. To maintain body temperature during anesthesia, water at 37° C. was circulated through the metal platform on which the animal rested.

The source of radiation was an intermediate pressure mercury arc equipped with a photocell and an integrating counter for monitoring the radiation. Essentially the same device was used in earlier studies, and has been described elsewhere (*2*, *3*). The mercury arc was placed at a distance of 44 cm., and mounted in such a position that the direct beam impinged normally upon the quartz surfaces that covered the ears. The exposures to ultraviolet light lasted only a few minutes, and placing the mouse in the holder required a few more. Even with the highest doses employed, the whole operation could be carried out in less than 20 minutes, during which anesthesia could be safely maintained.

For consistency with earlier studies on carcinogenesis, only adult male albino mice of strain A were used. The animals weighed between 20 and 30 gm. They were kept in separate cages in a dark room before and after irradiation to stabilize conditions by minimizing photorecovery (*4*) and the effect of light on mitotic activity.

In most of the studies described here, the 2 ears were given different doses of ultraviolet light, the right ear receiving 2.4×10^6 ergs cm.$^{-2}$, the left ear 1.2×10^6 ergs cm.$^{-2}$ of radiation, of wavelengths 0.313 μ and shorter (*2*, *3*).

In most experiments the anterior half of both ears was shielded from the radiation. After various numbers of days the animals were killed and the ears fixed in Gregg's modification of Worcester's fixative (*5*), double embedded in paraffin and celloidin, sectioned at 10 μ, and stained with hematoxylin and eosin. The areas that had been directly exposed to ultraviolet light were always compared grossly and histologically with adjacent shielded areas of the same ear. To make sure that none of the changes was due to pressure of the quartz slide, a control experiment was performed in which the ears were placed under the slide for an appropriate time under the usual conditions, but without irradiation. No deviation from the normal was seen.

In order to limit scratching of the ears to a minimum, the claws were trimmed off, and the animals were bedded on soft paper.

Results

The gross changes in the ear follow a similar but quantitatively different pattern according to the dose. In neither of the 2 doses studied was there any obvious change during the first day after irradiation. With the higher dose, erythema appeared shortly after the first day, almost always involving the whole ear. Usually within a few days the erythema had faded on the shielded part but remained on the irradiated area, so that a more or less distinct line of demarcation was seen. With the lower dose, the erythema appeared about the same time and tended to be restricted to the exposed area.

In sections of fixed tissue, the engorgement of the vessels that was manifested by the gross erythema was not easily detected, but at the end of about 3 days there was an increase in the number of stained nuclei in the dermis, which presumably represented migration of leukocytes out of the vessels. Within the first few days the ear was thickened in the irradiated part. This was seen in sections cut from fresh tissue, but was less easily detected in the fixed and sectioned material. Presumably, at this early stage the swelling represents principally edema that accompanies changes in the vessels. In the mouse ear, much of the ultraviolet radiation penetrates to the vessels in the dermis and may affect them directly to a much greater extent than occurs in human skin in which the epidermis is thicker and in which very little of this radiation reaches the dermis (*6*). There may be some temporary inhibition of dilation of the vessels after the irradiation, as is produced in human skin by massive doses of the longer wavelengths of the sunburn spectrum which penetrate to the dermis (*7*).

A general idea of the histological changes in the exposed area of the ear is given by the series of photomicrographs in figures 2 to 7, where figure 2 represents a section of normal skin and figure 3 the area of transition from normal to irradiated skin, at the end of 5 days. Figures 4 to 7 show examples of the condition of the irradiated skin at various times thereafter.

The epidermis of normal ears is 2 or 3 cells in thickness, whereas some days after irradiation it may be 10 or more cells thick. At this time the cells of the lowest layer appear essentially normal, though possibly swollen to some extent. Above this layer there are definitely swollen cells with nuclei that may be either swollen or pyknotic; presumably these are moribund cells. Superficially to this there appear some cells in which the nuclei are not apparent and above these a thick layer of condensed and flattened cells containing parakeratotic granules. A basal cell layer is difficult to distinguish in either the normal or irradiated epidermis.

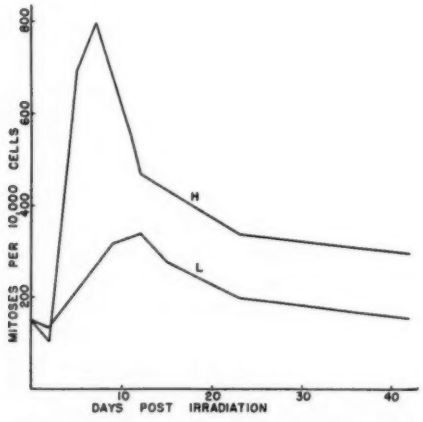
Although the greatest thickening of the epidermis is found on the upper surface of the ear which is directly exposed to the ultraviolet radiation, a definite thickening of the epidermis also occurs on the under surface of the corresponding area. It is to be noted from figure 3 that the change in thickness of the epidermis, as one passes from the irradiated area to that protected by the opaque shield, is very gradual, seemingly more than would be predicted on optical grounds alone. These changes will be discussed

further with regard to the incidence of mitosis in the various areas of the epidermis.

Small, quite sharply localized inflammatory lesions appear, involving both epidermis and dermis, which cannot be detected grossly within the first few days. These increase in number and extent and may become grossly observable as the maximum response is approached. As indicated in figure 4, the dermis in the immediate vicinity of these lesions is heavily infiltrated with leukocytes and fibroblasts, but that of the areas between the lesions is infiltrated to a much less degree. The basement membrane may break down and the epidermis may be greatly reduced in thickness, or absent at the site of the lesion, but elsewhere the epidermis remains intact and thickened over the greater part of the irradiated area. At times there is considerable exudate from the lesions as indicated by crusts that form over them, which extend to some extent over the adjacent areas. There is desquamation of the surface of the ear, representing in part these crusts, but no doubt including some corneum. The dermis thickens markedly, but the nature of the underlying changes is difficult to analyze. The number of sebaceous glands is reduced during this period, and hair follicles also begin to disappear. Downgrowths of epidermal tissue into the dermis appear, as shown in figure 5; the nature of these is uncertain—they possibly represent altered hair follicles.

Similar histological changes occur with the high and the low doses, though in general they are more marked in the former. Hyperplasia is more pronounced with the high dose.

A general healing process, taking place more rapidly with the lower dose, follows the events described. The thickness of the epidermis is reduced, epidermal hyperplasia decreases, and the hypertrophy of the



TEXT-FIGURE 1.—Incidence of mitosis in epidermis of the mouse ear directly exposed to ultraviolet radiation. H, curve describing incidence of mitosis following a dose of 2.4×10^6 ergs cm.$^{-2}$; L, curve for 1.2×10^6 ergs cm.$^{-2}$ These doses are based on wavelengths ≥ 0.313 μ, emitted by an intermediate pressure mercury arc (*2*). (Counts were made by G. A. S.)

cells disappears. The lesions heal and the crusts disappear, so that within a few weeks the ear returns to normal in most respects, both epidermis and dermis approaching their preirradiation dimensions. Sebaceous glands are still absent, as late as 39 days, in the area directly exposed to the radiation, as are hair follicles, for the most part, though both are still present on the under surface. Stages in the healing process are illustrated in figures 6 and 7, which represent sections from ears taken at 19 and 39 days after irradiation, respectively.

Although changes occur in the dermis and epidermis of the under-surface of the exposed area at the height of the response, these are much less severe than on the side directly exposed to the radiation, and, at the end of the healing process, the under part of the ear cannot be distinguished from the normal. For example, the sebaceous glands appear normal here at the end of 39 days (fig. 7), though they are absent from the upper side of the exposed area. The cartilage shows little or no alteration, though it may be displaced in areas of lesion or intense hyperplasia.

With still higher doses of ultraviolet radiation, large areas of the ears may be lost by necrosis.

Incidence of Mitosis

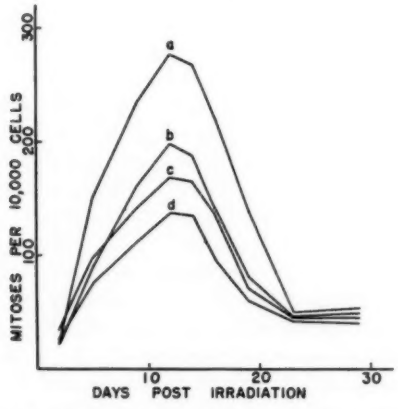
An index of the degree of hyperplasia of the epidermis should be given by the incidence of mitosis. The determination of mitotic indexes is somewhat complicated in the present study by a number of factors. The presence of the lesions on the exposed surface makes the selection of the appropriate areas, in which the counts are to be made, somewhat arbitrary. The gradual transition in thickness from irradiated to approximately normal epidermis offers a problem in comparing different regions of the ear. The decision, as to which cells are to be considered normal and which moribund, is somewhat less difficult, but offers another uncertainty. The count is best based on the number of intact nuclei (pyknotic nuclei and those in interphase without intact membranes being excluded), rather than on the number of cells. Since subjective judgment is involved in all these decisions, as well as with regard to the stages of mitosis to be counted, it is not surprising that counts made by 3 observers differed somewhat. All 3 obtained results in agreement, however, in regard to essential features of the course of the change in incidence after irradiation. In text-figures 1 and 2, the counts were made by a single observer and should be self-consistent.

Text-figure 1 compares the incidence of mitosis in the exposed area of the skin, for the high and low doses. It is seen that the count rises higher in the high dose and reaches its maximum earlier. After passing the maximum, the curves die away gradually, the curve for the lower dose reaching virtually normal incidence by 42 days post irradiation, whereas that for the high dose remains well above normal at this time. An apparent small decrease in incidence at 2 days is probably within the limits of error.

Text-figure 2 represents the change in incidence of mitosis in 4 different areas of ears exposed to the lower dose: *a*, the area directly exposed to

the radiation; *b*, the underside of the same region; *c*, the upper surface of the area covered by the opaque screen beyond the transition zone; and *d*, the undersurface of this same area. All 4 curves follow a similar course, reaching their maxima at the same time; the magnitude of the change occurs in the order of the areas listed. This is a somewhat troubling finding. In the first place, it is clear that one cannot use the shielded area of the ear as a control of the changes in the irradiated portion. But the explanation of this effect presents a still more bothersome problem.

Measurements of transmission of ultraviolet radiation indicate that, although a considerable fraction of the radiation of wavelengths near 0.3 μ passes through the epidermis of the ears, very little radiation of any effective wavelength reaches the epidermis of the opposite surface (*6*). The absence of marked changes in the cartilage and in the dermis on the underside (*note* particularly the sebaceous glands) also suggests that the amount of radiation reaching the epidermis of the undersurface is not great. Moreover, the definite increase of the incidence of mitosis in the areas shielded by the opaque screen suggests some explanation other than direct action of radiation at the undersurface of the ear. Perhaps the most telling argument against direct action of radiation at the undersurface is that the curve for incidence of mitosis reaches its maximum in this area at the same time as the corresponding curve for the irradiated area, as is shown in text-figure 2. The curves in text-figure 1 show that the maximum incidence of mitosis is reached at different times for different doses, and if the increase of mitosis in the epidermis of the undersurface were the result of a dose of radiation reduced in passing through the ear, curve *b* in text-figure 2 should have its maximum later than curve *a*. The correspondence of the time of the maxima in all the curves in text-



TEXT-FIGURE 2.—Incidence of mitosis in epidermis of 4 areas of a mouse ear exposed to ultraviolet radiation: *a*, the area directly exposed to the radiation; *b*, the underside of the same region; *c*, the upper surface of an area covered by an opaque shield, beyond the transition zone; *d*, the undersurface of this same area. The dose of radiation was the same as for curve L in text-figure 1. (Counts were made by J. R. D.)

figure 2 argues strongly against a direct effect of radiation except in the area directly exposed.

Such arguments lead to the idea that the effect on hyperplasia of the epidermis, outside the area directly exposed, is mediated by a diffusible substance, but it is difficult to assign channels for its diffusion. For the time being we are unable to explain this apparent "effect at a distance."

Discussion

The changes in the ear following a single dose of ultraviolet radiation are complex, involving a variety of tissues and cell types. At the present time it would be unwise to attempt a thorough discussion of quantitative relationships or of cytological details. In general, the reaction is inflammatory, but it seems somewhat unusual in character, perhaps because the immediate changes caused by the radiation are limited to a relatively thin layer within which the initiating photochemical changes take place.

The occurrence of localized, open lesions in the irradiated ears during the period of most active change in the tissues was not anticipated, and we are not yet altogether able to explain this finding. Although the exposed surface of the ear receives virtually the same incident dose of ultraviolet light, it is possible that the amount reaching the viable tissues varies from place to place because of differences in thickness of the corneum, which is a strong absorber of this radiation (*6*, *7*). It is notable that the lesions do not occur as a rule on the outer margin of the ear; perhaps the corneum is thicker here, or other anatomical and optical differences may be concerned.

There is, of course, the possibility that the open lesions result from the mouse scratching its ear, despite continuing precautions taken to prevent this. But, if scratching were the cause of the lesions, its effect could hardly be as accurately localized to the irradiated parts as is true. Finally, in 4 experiments the animals were completely immobilized for a sufficient time after irradiation for lesions to appear—5 days in 2, and 8 days in 2. In all experiments lesions occurred which were clearly not the result of scratching.

With regard to the relationship between the present studies and those on carcinogenesis (*1*, *8*, *9*), it should be pointed out that the doses used in the 2 kinds of experiments are not directly comparable in quantitative terms, though the type of source and method of measurement were the same. In the carcinogenic studies the mice were not immobilized but were allowed to move about freely within small cages (*2*). Thus the ears were not presented in a fixed position with regard to the source, as in the present study, and for a single given exposure, the radiation received by the ears must have been considerably less. Since, in the studies on carcinogenesis, the mice were subjected to repeated doses, and insofar as could be determined their movements were random, the dosage should have been comparable within the particular set of studies, as the consistency of the results indicates (*8*, *9*), but the measured dose must have been effectively less than under the conditions of the present experiments.

Let us attempt a rough comparison of the effective dosage under these 2 conditions of irradiation. The area presented to a normal beam of light is different for the ear, in its natural curved position, from the area of the ear when flattened under a quartz plate. Measurements of the 2 ears of 1 of these mice gave 3.3 $cm.^2$ for the average profile in the normal state, and 4.7 $cm.^2$ for the area when flattened out. Thus, in a beam at normal incidence, the unflattened ear would receive $3.3/4.7 = 0.7$ times as much incident radiation as the flattened ear. This should represent the difference under these 2 conditions if the mice were maintained in the indicated position relative to the source; but in the carcinogenic studies the animals were free to move about. Let us assume that in the latter case the mice changed position randomly by movement around the longitudinal axis; we may then, for purposes of analysis, assume that they rotated around this axis at a constant rate. If we assume that the undersurface of the ear is shielded at all times by the body of the mouse, the outer surface should be presented to the radiation during only one half the time, and the average angle presented to the source being 45°, the correction $0.5 \times \cos 45°$ should be made, *i.e.*, 0.5×0.7. Thus, on the basis of the assumptions made, the dosage received by the mice in the carcinogenesis experiments should be $0.7 \times 0.5 \times 0.7 = 0.25$ that received in the present experiments, the energy incident at the average plane of the ears being the same.

The conditions assumed for the carcinogenesis experiments are quite artificial and probably minimize the difference in the 2 studies. The animals move with regard to the transverse axis as well as the longitudinal, and in a quite irregular manner, though apparently at random (*2*). But it seems reasonable to conclude that the same measured dose is several times as effective in the present experiments as in those on carcinogenesis. This would still place the doses used here among the highest employed in the carcinogenic studies, *i.e.*, single doses (*9*). Under the circumstances it would appear that for the greater range of the individual doses used to induce cancer, the histological changes resulting from the first dose were slight. This fits with less well-controlled observations in this regard at the time the carcinogenic studies were made.

Without exploration of the effect of repeated doses, the present studies can provide little evidence that is directly applicable to carcinogenesis. One exception may be cited. Grady *et al.* (*10*) found only 1 sebaceous-gland carcinoma among over 600 tumors of the ear induced by repeated doses of polychromatic ultraviolet radiation. The disappearance of the sebaceous glands after a single dose of the same type of radiation would seem to account for the paucity of such tumors.

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PLATE 91

FIGURE 1.—Apparatus for holding mouse during exposure of ears to ultraviolet radiation. (Only 1 ear of mouse has been mounted in position for radiation; in practice, both ears are similarly placed.) T, metal platform through which water at 37° C. is circulated; P, metal plate against which mouse ear is flattened by means of quartz plate; L, microscope slide made from high quality fused quartz. Quartz plate is held in place by a rubber band. Metal plate is mounted on a triangular metal block, which holds it at an angle of 45° to platform. Shield, O, removed while ears are being placed in position, protects eyes of animal from radiation.

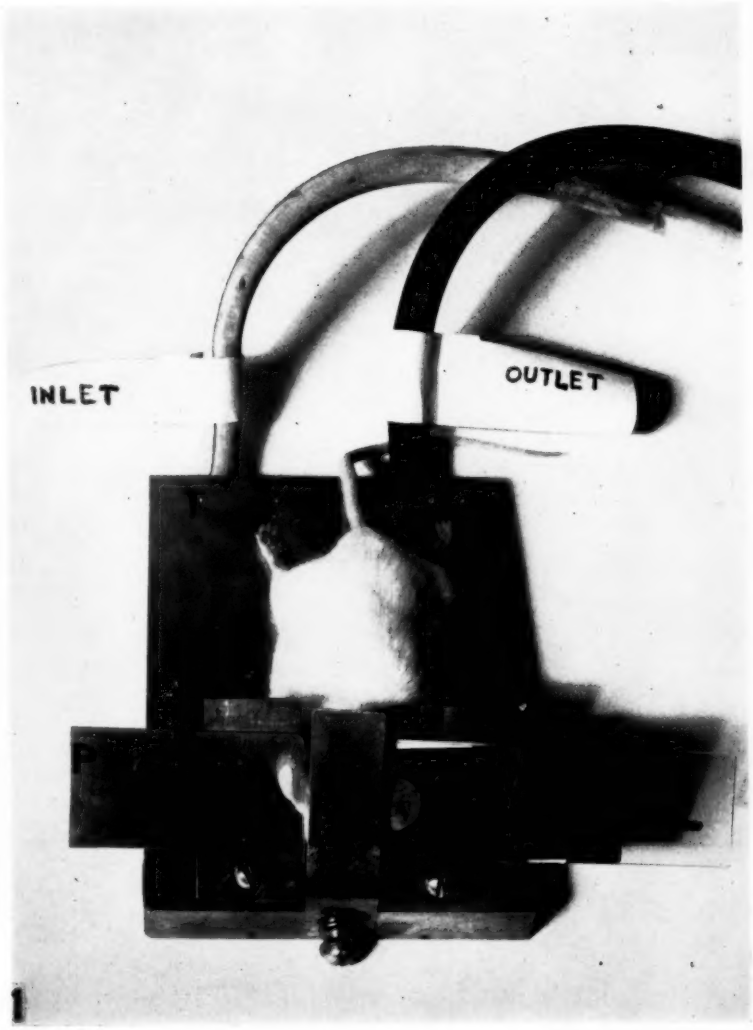

INLET
OUTLET
1

PLATE 92

Photomicrographs of sections of mouse ears. Hematoxylin and eosin. × 125

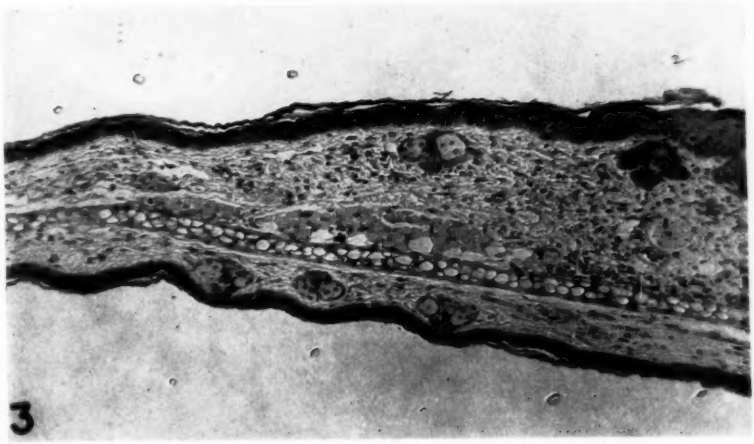
FIGURE 2.—Normal mouse ear, for comparison.

In figures 3 to 7, half of upper surface of ear was exposed to ultraviolet radiation, dose 2.4×10^6 ergs cm.$^{-2}$ Irradiated surface is uppermost in all figures. Under portion of ear is essentially normal in regard to changes that can be seen in photomicrographs at this magnification, except for slight thickening of the epidermis. It thus provides a basis of comparison with changes in upper portion.

FIGURE 3.—Five days after irradiation. Transition from irradiated skin (*right*) to unirradiated skin (*left*). *Note* gradual transition from thickened to normal dimensions in upper portion of ear.

FIGURE 4.—Five days after irradiation. Upper surface shows open lesion at *left*, with loss of definition of basal membrane, and heavy leukocytic invasion concentrated in region of lesion. Intact part of epidermis may be slightly thickened; dermis is clearly thickened, but there are less leukocytes in this region.

2



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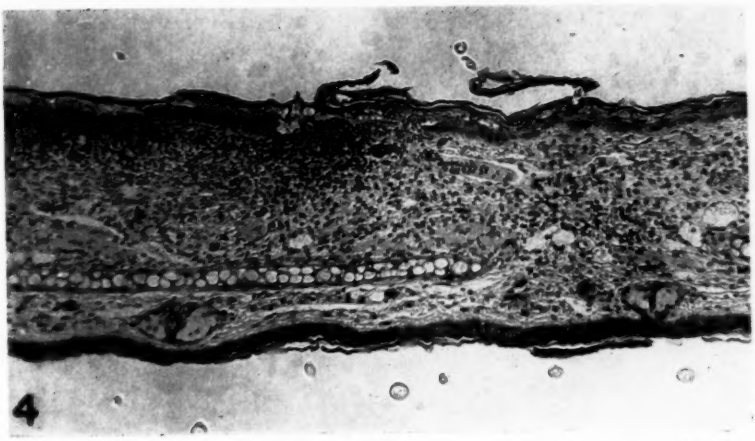


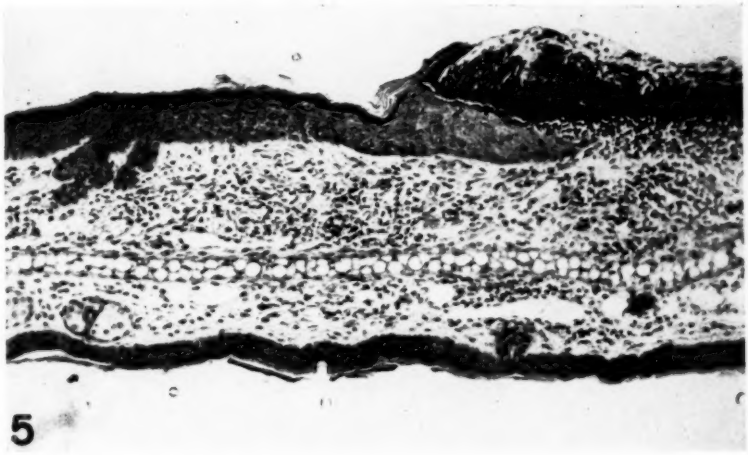
4

PLATE 93

FIGURE 5.—Eleven days after irradiation. Upper surface of ear shows open lesion on *right*, with heavy, overlying crust. Leukocytes are plentiful near lesion. Epidermis is greatly thickened near lesion and in adjacent irradiated area. *Note* downgrowth of epithelium on *left*, thickening of dermis, and absence of sebaceous glands.

FIGURE 6.—Nineteen days after irradiation. Upper surface shows no open lesions. Both epidermis and dermis are thinner than in figure 5. Sebaceous glands are absent. *Note* clearly defined parakeratotic layer at upper surface, appearing as dark, compressed zone between viable epidermis and corneum, and its virtual absence at lower surface.

FIGURE 7.—Thirty-nine days after irradiation. Upper surface of ear has somewhat thickened epidermis with heavy parakeratotic layer. Dermis is thickened, with dense collagen. Sebaceous glands are absent and very few hair follicles remain. Lower surface appears entirely normal; *note* particularly presence of sebaceous glands.



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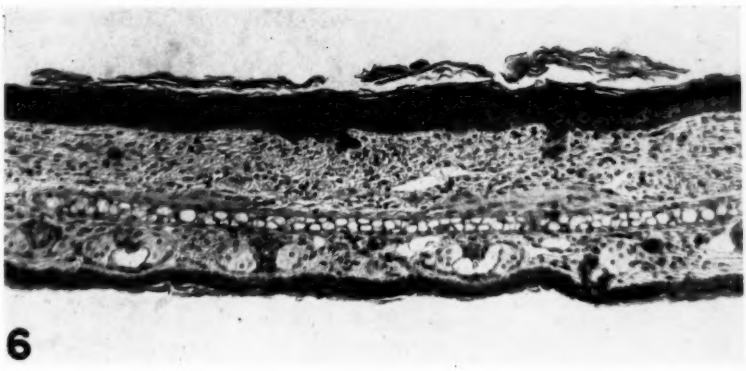
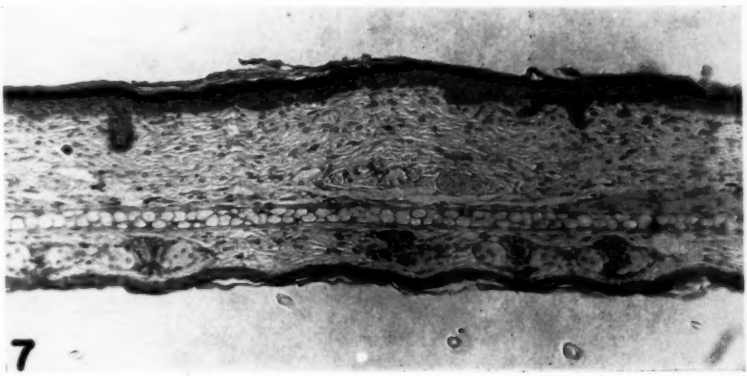


6



7

Occurrence of Tumors, Particularly Mammary Tumors, in Agent-Free Strain C3HeB Mice [1, 2]

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Summary

The development of tumors has been studied in mice of substrain C3HeB which was produced by transfer of fertilized ova from strain C3H to strain C57BL. Mammary tumors occurred in 4 percent of virgin females of substrain C3HeB at an average age of 20.7 months, in 54.8 percent of breeding females at an average age of 19.2 months, and in 74.4 percent of force-bred females at an average age of 17.9 months. Mammary tumors occurred in 22.4 percent of C3HeB males, treated with diethylstilbestrol, at an average age of 15.0 months. Hepatomas occurred more frequently in animals of substrain C3HeB than have been observed previously in any other inbred strain. They were found in 58.6 percent of virgin females at an average age of 24.1 months, in 30.3 percent of breeding females at an average age of 21.0 months, in 37.8 percent of force-bred females at an average age of 20.5 months, in 55.1 percent of males, treated with diethylstilbestrol, at an average age of 23.3 months, and in 90.5 percent of breeding males at an average age of 21.4 months. Ovarian tumors occurred frequently in the females of substrain C3HeB: in 47.5 percent of virgin females at an average age of 24.3 months, in 37.4 percent of breeding females at an average age of 21.5 months, and in 29.3 percent of force-bred females at an average age of 21.6 months.—
J. Nat. Cancer Inst. 22: 995–1002, 1959.

In studies of mammary tumors there is often a need for a strain of mice that does not have the mammary-tumor agent but is genetically susceptible to it. One such strain is a substrain of strain C3H, known as C3Hf, upon which extensive studies of the development of mammary tumors have been made in this laboratory (*1–3*). A second such strain, which will be discussed here, is substrain C3HeB (*4*), produced by transfer of fertilized ova from strain C3H to strain C57BL.

Materials and Methods

The colony of strain C3H mice, from which substrain C3HeB (hereinafter referred to as C3He) was originated, was obtained from Dr. W. E.

[1] Received for publication December 2, 1958.

[2] Part of the material in this paper was presented at the Seventh International Cancer Congress, London, July 6–12, 1958.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Heston in 1952. His line was separated in 1941 from the colony of Dr. H. B. Andervont, whose line, in turn, had been obtained from Dr. L. C. Strong in 1930. Up to the present time, 223 (94.1%) of 237 breeding females of the strain C3H colony have developed mammary tumors at an average age of 8.3 months, and 14 (5.9%) have died without mammary tumors at an average age of 7.3 months. Strain C57BL mice, a low-tumor strain, were obtained from Dr. Heston in 1949. His line had been obtained from Dr. Andervont in 1945. No mammary tumors have been observed in any of the lines mentioned.

Two days after observation of vaginal plugs, fertilized ova were transferred from the oviducts of strain C3H breeding females to the uteri of strain C57BL females. The technique of transferring ova in mice has been described by Fekete and Little (*5*). At birth, the young were nursed by their foster mothers and were reared by them to weaning age. Seventeen transfers involving a total of 63 fertilized ova were made. Six of the transfers were successful and resulted in a total of 7 young (4 males and 3 females). The colony of substrain C3He was established from a brother-sister pair of these mice.

Descendants of these C3He mice were placed in 3 groups, according to the treatment received, and were compared with females of generations F_1 through F_7 of the breeding colony. Group 1 was composed of females kept as virgins. Group 2 was composed of females that were force-bred and isolated when pregnant. After birth of the litter, the young were discarded, and the mothers were returned to the breeding cages at once. This procedure, described by Bagg (*6*), was continued throughout the breeding period of the females. Group 3 consisted of C3He males. Cholesterol pellets, weighing 6 to 8 mg. and containing 10 percent diethylstilbestrol, were implanted subcutaneously in the right axilla of the males when they were 1 to 2 months of age. The various groups of mice were observed at regular intervals for the development of mammary tumors.

The mice were kept in 7 × 11 × 5 inch plastic cages, 8 to a cage, and were fed NCI pellets [4] and tap water *ad libitum*. The animals were killed and autopsied when they developed tumors or became moribund. Tissues were fixed in Fekete's modification of Tellyesniczky's fluid (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part), sectioned, and stained with hematoxylin and eosin.

Results and Discussion

Data on the occurrence of mammary tumors in the various groups of substrain C3He mice are presented in table 1. Mammary tumors developed in 4 percent of virgin females, in 54.8 percent of females of the breeding colony, in 74.4 percent of force-bred females, and in 22.4 percent of males, treated with diethylstilbestrol. These data can be compared

[4] The NCI pellets, formerly referred to as Derwood pellets, are prepared by Herbert Bryant, Inc., Gaithersburg, Md., and are of the following composition: skim-milk powder (human grade), 23.29%; ground whole hard wheat, 65.10%; dried debittered Brewer's yeast, 4.00%; corn oil, 5.80%; sodium chloride (iodinated), 1.40%; iron citrate, U.S.P. No. 7 powder, 0.13%; shark-liver oil (4.8 million units vitamin A per pound), 0.20%; and Delstrol (900,000 units vitamin D per pound), 0.08%.

TABLE 1.—*Occurrence of mammary tumors in C3He mice*

Group	Number of animals	Average No. of litters	Tumorous mice			Nontumorous mice	
			Number	Percent	Average age (*months*)	Percent	Average age (*months*)
Females (virgins)	99	—	4	4.0	20.7	96.0	23.4
Females (breeders)	155	4.8	85	54.8	19.2	45.2	19.6
Females (force-bred)	82	6.1	61	74.4	17.9	25.6	22.1
Males (diethylstilbestrol-treated)	98	—	22	22.4	15.0	77.6	22.9

with data obtained previously for C3Hf mice (*1–3*). Mammary tumors developed in 2 percent of virgin females of substrain C3Hf, in 38 percent of breeding females, and in 20 percent of males, treated with diethylstilbestrol. In generations F_6 through F_{25} of this same line of C3Hf, mammary tumors developed in 22 percent of breeding females. Data for the 2 substrains corresponded quite closely except for the greater incidence of mammary tumors in breeding females of the C3He substrain. In another line of C3Hf mice, originated by Andervont (*7*), mammary tumors developed in 3 percent of breeding females of generations F_1 through F_{10}. In this same line of C3Hf, maintained by Heston (*8*) for 6 generations (F_{26}–F_{31}), mammary tumors developed in 27 percent of breeding females. This difference in mammary-tumor incidence may be the result of differences in breeding systems and in lifespan of the animals in the 2 laboratories. Mammary tumors have developed in 29 percent of breeding females of generations F_1 through F_7 of a doubly foster-nursed line, designated C3Hff, and in 51 percent of breeding females of generations F_1 through F_5 of a second line of C3Hf, designated C3Hf/2. The incidences of mammary tumors for the groups of C3He mice were similar to those obtained by Boot and Mühlbock (*9*) for their substrains C3Hf and C3He (produced in their laboratory from strain C3H mice obtained from Dr. Heston in 1951). The mammary-tumor incidence for their substrain C3Hf virgins was 11 percent, for breeders, 42 percent, and for force-bred females, 63 percent; for substrain C3He virgins the incidence was 6 percent, for breeders, 47 percent, and for force-bred females, 56 percent. Boot and Mühlbock (*9*) have also studied the incidences of mammary tumors in gonadectomized males of substrains C3Hf and C3He treated with estrogen in the drinking water. Mammary tumors developed in 37 percent of substrain C3Hf males on a low dosage of estrogen and in 71 percent of substrain C3Hf males on a high dosage. In the C3He males on a low dosage, 61 percent developed mammary tumors and on the high dosage, 69 percent developed mammary tumors. In all of these groups of males the incidences of mammary tumors were higher than the 22.4 percent observed in the substrain C3He males in the present study, and the difference can be explained by the differences in the treatment which the animals received. However, the incidence ob-

served in the males of substrain C3He was like that of 20 percent observed in males of substrain C3Hf similarly treated (*3*).

It is well known that mammary tumors will develop in mice in the absence of the mammary-tumor agent (*1, 7–11*). In animals without the agent, one of the factors that has a strong influence on the incidence of mammary tumors is an increase in hormonal stimulation resulting from an increase in number of litters produced. Substrain C3He females of the breeding colony produced an average number of litters of 4.8 with an incidence of mammary tumors of 54.8 percent, while force-bred females produced an average of 6.1 litters with an incidence of 74.4 percent (*see* table 1). It has been observed by Heston (*8*) that the more litters a female of substrain C3Hf produced, the more likely she was to develop a mammary tumor. In table 2, data for the occurrence of mammary tumors in substrain C3He are arranged according to the number of litters each female produced, and indicate a similar trend for this substrain. Boot and Mühlbock (*9*) have observed an average number of litters of 5.3 for breeding females and 5.6 for force-bred females of substrain C3Hf, with mammary-tumor incidences of 42 and 55 percent, respectively; and of 5.0 for breeding females and 5.4 for force-bred females of substrain C3He, with mammary-tumor incidences of 67 and 61 percent.

Mammary tumors of various types, classified according to Dunn (*12*), have been observed in C3He mice [5] and are listed in table 3. In virgin females the mammary tumors were adenocarcinomas of type B (sheets of cells or chords of cells separating cystic areas). In breeding females, adenocarcinomas of type A (acinar structure) and adenoacanthomas (glandular and epidermoid tissue) occurred about equally, and adenocarcinomas of type B occurred in about one fourth more animals than either of the above 2 types. In force-bred females, 3 types (adenocarcinomas of types A and B, and adenoacanthomas) occurred with about equal frequency. In each of these 2 latter groups there were a few miscellaneous tumors and a few mixed tumors. In males, treated with diethylstilbestrol, the numbers of adenocarcinomas of type A and of type B were about equal.

Mention should be made of the secretory activity observed in histologic sections of some of the mammary tumors occurring in C3He mice (table 3). It was observed in 1 of 4 tumors in virgin females, in 11 of 96 tumors in breeding females, in 8 of 74 tumors in force-bred females, and in 15 of 22 tumors in males treated with diethylstilbestrol. This type of activity has been described by Foulds (*13*) as occurring in mammary tumors of (C57BL × RIII)F_1 females in late stages of pregnancy and of (C57BL × RIII)F_1 males carrying diethylstilbestrol pellets, and has been discussed by Dunn (*12*) in her morphologic study of mammary tumors in mice.

One outstanding observation in this study was the high frequency of hepatomas in the C3He mice (table 4). They developed in 58.6 percent of virgin females, in 30.3 percent of breeding females, in 37.8 percent of

[5] The author is grateful to Dr. Thelma B. Dunn, pathologist, of the National Cancer Institute, who confirmed the diagnosis of many of the tumors observed.

TABLE 2.—*Occurrence of mammary tumors in females of substrain C3He according to number of litters each produced*

Number of litters	Number of animals	Mammary tumors		Average age females died without tumors (*months*)
		Percent	Average age (*months*)	
0	99	4. 0	20. 7	23. 4
1	3	33. 3	25. 0	22. 0
2	6	16. 6	15. 0	23. 4
3	21	47. 6	21. 1	19. 6
4	35	62. 9	18. 3	20. 2
5	42	59. 5	19. 4	20. 6
6	31	51. 6	19. 3	17. 8
7	10	40. 0	19. 8	16. 0
8	5	80. 0	19. 0	22. 0
9	1	100. 0	14. 0	—
10	—	—	—	—
11	1	100. 0	13. 0	—

TABLE 3.—*Classification of mammary tumors in C3He mice*

Type	Number in virgin females	Number in breeding females	Number in force-bred females	Number in diethylstilbestrol-treated males
Adenocarcinoma—type A	—	26(8)*	21(5)	10(9)
Adenocarcinoma—type B	4(1)	35(3)	23(3)	12(6)
Adenoacanthoma	—	27	23	—
Miscellaneous	—	7	3	—
Mixed	—	1†	4‡	—
Totals	4(1)	96(11)	74(8)	22(15)

*Numbers in parentheses indicate the number of tumors exhibiting secretory activity.
†Type B + adenoacanthoma.
‡Three type A + adenoacanthoma, 1 type B + adenoacanthoma.

TABLE 4.—*Occurrence of hepatomas in C3He mice*

Group	Number of animals	Hepatomas		
		Number	Percent	Average age (*months*)
Females (virgins)	99	58	58. 6	24. 1
Females (breeders)	155	47	30. 3	21. 0
Females (force-bred)	82	31	37. 8	20. 5
Males (diethylstilbestrol-treated)	98	54	55. 1	23. 3
Males (breeders)	21	19	90. 5	21. 4

force-bred females, in 55.1 percent of males, treated with diethylstilbestrol, and in 90.5 percent of breeding males. This is the highest occurrence of spontaneous hepatomas ever reported for any strain of mice (*1*, *3*, *14*). The reduction in incidence of hepatomas in males, treated with diethylstilbestrol, has been observed in substrain C3Hf (*3*).

Various other tumors have been observed in C3He mice. Data for those occurring in females are presented in table 5 and for those in males in table 6. The high incidences of ovarian tumors (consisting mainly of granulosa-cell tumors and tubular adenomas) were most striking. Ovarian tumors occurred in 47 (47.5%) of virgin females, in 58 (37.4%) of breeding females, and in 24 (29.3%) of force-bred females. Table 5 shows a total of 49 ovarian tumors in virgin females, 60 in breeding females, and 25 in

TABLE 5.—*Occurrence of tumors in female mice of substrain C3He*

Type of tumor	Virgin females*		Breeding females†		Force-bred females‡	
	Number with tumors	Average age (*months*)	Number with tumors	Average age (*months*)	Number with tumors	Average age (*months*)
Granulosa-cell tumor of ovary	20	24. 0	36	20. 6	11	21. 5
Tubular adenoma of ovary	25	24. 5	22	22. 7	12	21. 7
Luteoma of ovary	2	24. 0	1	23. 0	1	19. 0
Mixed tumor of ovary	2	23. 5	1	23. 0	—	—
Unclassified tumor of ovary	—	—	—	—	1	21. 0
Sarcoma—subcutaneous	7	22. 1	3	25. 7	1	24. 0
Sarcoma of uterus	3	22. 3	—	—	—	—
Hemangioendothelioma	2	24. 5	8	21. 8	1	24. 0
Pulmonary tumor	14	24. 6	8	23. 1	3	15. 7
Polyp of uterus	4	21. 5	2	20. 5	1	17. 0
Reticulum-cell neoplasm—type A	2	21. 5	1	21. 0	—	—
Reticulum-cell neoplasm—type B	—	—	3	21. 7	—	—
Lymphocytic neoplasm	1	26. 0	—	—	—	—
Plasma-cell neoplasm	—	—	—	—	1	19. 0
Squamous-cell carcinoma	—	—	1	11. 0	—	—
Myoepithelioma	—	—	—	—	1	19. 0
Pheochromocytoma	—	—	—	—	1	23. 0
Papilloma of skin	—	—	1	21. 0	—	—

*Total number: 99. †Total number: 155. ‡Total number: 82.

force-bred females. The fact that 2 virgin females, 2 breeding females, and 1 force-bred female had coexisting tumors of different types in alternate ovaries accounts for the differences in the figures from those cited above. The differences in the incidences of ovarian tumors in the 3 groups can probably be explained by the fact that the virgins lived longer and more of the force-bred females died early with mammary tumors. Since both hepatomas and ovarian tumors occurred so frequently, the possibility that there was an association between the 2 was explored. Both types were found to be randomly distributed throughout the 3 groups. Ovarian tumors have been reported to occur only rarely in mice (*15*, *16*) except in strain CE. Woolley and Little (*17*) have observed a 33-percent inci-

TABLE 6.—*Occurrence of tumors in male mice of substrain C3He*

Type of tumor	Treated with diethylstilbestrol*		Breeders†	
	Number with lesion	Average age (*months*)	Number with lesion	Average age (*months*)
Sarcoma	4‡	24. 0	—	—
Hemangioendothelioma	3	25. 6	—	—
Pulmonary tumor	10	24. 3	5	22. 8
Reticulum-cell neoplasm—type A	2	24. 0	—	—
Reticulum-cell neoplasm—type B	1	26. 0	—	—
Lymphocytic neoplasm	1	26. 0	—	—
Adrenocortical adenoma	1	27. 0	1	25. 0
Carcinoma of kidney	1	27. 0	—	—
Harderian-gland tumor	—	—	1	23. 0

*Total number of animals: 98.
†Total number of animals: 21.
‡Three subcutaneous sarcomas, 1 of mesenteric node.

dence of ovarian tumors in strain CE females, and Dickie (*18*) has observed a 52-percent incidence of ovarian tumors in CE backcross females. Heston (unpublished data) has observed an incidence of between 5 and 10 percent in a group of old C3Hf females which had been foster-nursed. The tumors were frequently small and occurred often in connection with cysts. Data on their occurrence alone or in association with cysts are presented in table 7. Ovarian cysts were often seen in females in which ovarian tumors did not occur. They were observed in 40 (affecting 1 ovary in 28 and both ovaries in 12) of 52 virgin females, in 51 (39 of 1 ovary, 12 of both) of 97 breeding females, and in 30 (24 of 1 ovary, 6 of both) of 58 force-bred females.

Pulmonary tumors were observed in a small number of animals of each experimental group. No other tumor occurred frequently enough to warrant any particular comment.

TABLE 7.—*Occurrence of ovarian tumors and ovarian cysts in C3He females*

Tumors and cysts in ovaries	Number in virgin females	Number in breeding females	Number in force-bred females
Tumor of 1 without cyst	2	1	2
Tumor of 1 without cyst and cyst of other	2	4	2
Tumor of 1 with cyst	12	17	9
Tumor of 1 with cyst and cyst of other	25	28	6
Tumors of 2 without cysts	—	1	—
Tumors of 2 with cyst of 1	3	1	—
Tumors of 2 with cysts of 2	3	6	5
No tumor and cyst of 1	28	39	24
No tumors and cysts of 2	12	12	6
No tumor and no cyst	12	46	28

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Rapid Proliferation of Sublines of NCTC Clone 929 (Strain L) Mouse Cells in a Simple Chemically Defined Medium (MB 752/1)[1,2]

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Summary

A chemically defined nutrient solution containing 40 components is described. In this solution, cells of sublines of NCTC clone 929 (strain L) mouse cells have been maintained for more than 30 passages in rapid proliferative growth, with the cell population increasing 7.5-fold to 8.5-fold (from an inoculum of 2 to 3 $\times$ 10^5 cells per ml.) in an 8-day period.—**J. Nat. Cancer Inst. 22: 1003–1017, 1959.**

A number of chemically defined nutrient media for NCTC clone 929 (strain L) cells (*1–8*) and for other cells (*1, 9–13*) has been described during the past few years. Most of these media permit, alone, only maintenance of the cell population (*1, 2, 9, 10*) or slow proliferation (*3–8, 11–14*). Supplementation with serum (*15–26*) or with some other undefined components, *e.g.*, protein, protein hydrolysates, or peptones (*16, 27–32*), has until the last few years generally been thought necessary to attain rapid growth, though Healy *et al.* (*7*) had a subline of strain L which in 7 days increased ninefold to tenfold in cell population in medium CMRL–858, a solution containing 62 components. McQuilkin *et al.* (*5*) and Sanford *et al.* (*14*), using medium NCTC 109 which contained 68 components, have maintained a subline (NCTC 2071) of this strain in continuous proliferation for several years at a lower rate, so that the cultures could be subdivided to form 2 daughter cultures every 7 days. It has been shown (*33*) that, with an optimum size of initial cell inoculum, NCTC 109 can support a 13.1-fold increase in 7 days. Both Parker's CMRL-858 and later media (*7, 17*) and Earle's NCTC 107 to 110 (*3–5, 14, 34*) contain several coenzyme preparations rather than the constituent active vitamins.

Since the development and description of fully defined maintenance media (*1*) and of a protein- and peptone-containing medium capable of supporting rapid growth (*29*), our efforts have been directed along two

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lines: 1) We have attempted to identify the unknown constituents which contribute to the proliferation-promoting activity of peptones (Waymouth and Matthews, in preparation); 2) we have systematically improved the defined part of the nutrient solution to the point where it alone is capable of supporting rapid and continuous growth of our sublines of clone 929 (strain L) cells.

The medium MB 752/1 described later is a chemically defined solution of 40 components, containing only salts, glucose, vitamins, amino acids, and the purine base, hypoxanthine. It is presented in the belief that this simple and easily prepared solution may prove useful to others for studies on the nutrition, metabolism, and growth of sublines of strain L, and may also, by demonstrating that rapid, continuous growth of one cell strain is possible in a simple medium, strengthen the resolution of those who seek comparable media for other cells, as well as help to convert those whose faith is still in complex growth factors.

Materials and Methods

The Cells and Their Cultivation

The sublines of strain L (NCTC clone 929) used in the main part of this study were initiated from culture 20 of clone 929, transfer generation 144 received in December, 1953, from Dr. Wilton R. Earle, and subsequently maintained in this laboratory in a succession of media of decreasing complexity. For the first few passages, the cells were carried, as in the parent laboratory, in media containing serum and embryonic extract. Subsequently they were grown in media containing serum but no embryonic extract; then in serum-free media such as ML 192/2 (*29*), containing protein and peptone; and, for more than a year before transfer to fully synthetic media, in peptone-containing but protein-free media such as ML 838/1. The composition of the defined part of the solutions was progressively improved during this time until it became possible to discard the peptone entirely, early in 1958. Because, during the history of this subline, it was thought that some "adaptation" to the defined type of medium might have occurred, a new subline of strain L cells was furnished by Dr. Earle, in August, 1958, which derived from a culture 929-371-302 that had been carried throughout its history, in the National Cancer Institute, in chick-embryo extract plus horse serum plus balanced salt solution (20:40:40) medium; these cells were transferred directly into the synthetic medium MB 752/1 from the complex biological medium.

All our strain L cells are maintained as stationary cultures in Carrel D-3.5 flasks, with renewal of the nutrient medium twice weekly. Antibiotics and mycostatics have not been used.

Growth Measurements

For quantitative estimation of the rate of proliferation, replicate cultures were prepared. The inoculum in each Carrel D-3.5 flask containing

1 ml. of medium ranged from 2.0 to 3.0 $\times$ 10^5 cells. The increase in cell population was followed by periodical measurements, in a microhematocrit, of the total volume of cells per flask (*35*).

The Nutrient Medium

The solution MB 752/1 has been developed by a series of modifications from medium ML 192/2 (*29*) and from protein-free modifications of it, and differs from ML 192/2 in the following respects:

1) Protein (bovine serum albumin, Armour's fraction V) and peptone (Difco Bacto-Peptone) are omitted.

2) The total amino acids (S/S5 of ML 192/2) are doubled, L-valine and L-isoleucine are substituted for the DL forms, and the basic amino acids L-lysine·HCl and L-histidine·HCl are increased from 3.75 to 15.0 mg. per 100 ml. and from 8.0 to 24.0 mg. per 100 ml., respectively.

3) Glutamine is increased from 5.0 to 35.0 mg. per 100 ml.

4) Cysteine·HCl is increased from 7.78 to 9.0 mg. per 100 ml.

5) Mg^{++} is increased from 0.8 mmole to 2.0 mmole.

6) The concentrations of thiamine·HCl, calcium pantothenate, riboflavine, and pyridoxine·HCl are doubled; that of folic acid is increased fourfold; and that of biotin reduced to one fifth. The following additional B vitamins have been included:

Choline·HCl	at 25.0 mg. per 100 ml.
Nicotinamide	at 0.1 mg. per 100 ml.
m-Inositol·$2H_2O$	at 0.1 mg. per 100 ml.
Vitamin B_{12}	at 0.02 mg. per 100 ml.

The composition of the solution and the method of preparation are shown in table 1. The *p*H of the medium is 7.4. A fresh solution is usually prepared each week. However, this and similar media have been kept for several weeks at +5° without loss of effectiveness.

Medium MB 752/1 differs in some important ways from our earlier media (*1*, *29*) and from other chemically defined media in current use. Some of these differences are tabulated (table 2) and will be discussed later.

Amino Acids

The importance of amino acids, in general, for the growth of cells was first demonstrated by Fischer (*36*), with an artificial mixture of amino acids to supplement a dialyzed medium. It is of interest, in view of the present results, that, for chick fibroblasts, Fischer (*37*) found lysine could be omitted for several days from his medium without reducing the outgrowth of his cultures. Cystine was especially important as a supplement to dialyzed plasma and embryonic extract (*37*). Kieler (*38*), also using dialyzed media and chicken fibroblasts, demonstrated clear requirements for arginine (optimum concentration 10 mg./100 ml.) and histidine (optimum 10 mg./100 ml.) and a marked stimulation of mitosis with lysine·2HCl at 25 mg./100 ml.

TABLE 1.—*Medium MB 752/1*

S1			S2			S3		
Materials	mg./100 ml.	mmole	Materials	mg./100 ml.	mmole	Materials	mg./100 ml.	mmole
NaCl	600	103	Thiamine·HCl	1. 0	0. 03	L-Lysine·HCl	24. 0	1. 42
KCl	15	2. 0	Calcium pantothenate	0. 1	0. 002	L-Histidine·HCl	15. 0	0. 80
$CaCl_2 \cdot 2H_2O$	12	0. 82	Riboflavine	0. 1	0. 003	L-Glutamic acid	15. 0	1. 02
$MgCl_2 \cdot 6H_2O$	24	1. 18	Pyridoxine·HCl	0. 1	0. 003	L-Threonine	7. 5	0. 64
$MgSO_4 \cdot 7H_2O$	20	0. 81	Folic acid	0. 04	0. 0008	L-Arginine·HCl	7. 5	0. 36
Dextrose	500	27. 8	Biotin	0. 002	0. 00008	L-Valine	6. 5	0. 55
Ascorbic acid	1. 75	0. 1	*m*-Inositol·$2H_2O$	0. 1	0. 005	L-Aspartic acid	6. 0	0. 46
Cysteine·HCl	9. 0	0. 57	Nicotinamide	0. 1	0. 008	Glycine	5. 0	0. 66
Glutathione	1. 5	0. 05	Vitamin B_{12}	0. 02	0. 00015	L-Proline	5. 0	0. 44
Choline·HCl	25. 0	1. 8				L-Leucine	5. 0	0. 38
Hypoxanthine	2. 5	0. 18				L-Methionine	5. 0	0. 34
Glutamine	35. 0	2. 38				L-Phenylalanine	5. 0	0. 30
Na_2HPO_4	30. 0	2. 11				L-Tyrosine	4. 0	0. 22
KH_2PO_4	8. 0	0. 59				L-Tryptophan	4. 0	0. 20
$NaHCO_3$	224. 0	26. 7				L-Isoleucine	2. 5	0. 19
						L-Cystine	1. 5	0. 006
						NaOH (to *p*H 7.4)		2. 5
The above ingredients, made up at double the stated concentrations in glass-distilled water, constitute stock solution S1. Prepare by first dissolving all the components except the buffer salts in about 80 percent of the water. Then add the buffer salts in a small volume of water and make up to the total volume. This procedure prevents the precipitation of Ca or Mg phosphates.			The stock solution of the above B vitamins, S2, is made up at 40 times the final concentration.			The stock solution of amino acids, S3, is made up at 10 times the final concentration.		

The stock solutions S1, S2, and S3 are sterilized by passage through Selas #03 filters. Solution S1 may be stored at +5° for up to 3 weeks. Solutions S2 and S3 keep indefinitely.

The nutrient solution MB 752/1 is prepared by adding to 37.5 ml. water, 50 ml. of S1 + 2.5 ml. of S2 + 10 ml. of S3 (total of 100 ml.).

TABLE 2.—*Comparison of some components in medium MB 752/1 and other synthetic media for strain L cells (mg./100 ml.)*

Components	MB 752/1	ML 192/2 (*29*)	Parker CMRL 1066 (*17*)	Earle NCTC 109 (*14*)	White WII (*2*)	Eagle (*19*)
L-Arginine·HCl	7. 5	3. 75	7. 0	2. 6	7. 8	1. 05
L-Histidine·HCl	15. 0	3. 75	2. 0	2. 0	2. 6	0. 2
L-Lysine·HCl	24. 0	8. 0	7. 0	3. 0	15. 6	0. 9
L-Glutamine	35. 0	5. 0	10. 0	13. 6	—	3. 0–7. 5
L-Cysteine·HCl	9. 0	7. 78	26. 0	26. 0	0. 1	—
L-Cystine	1. 5	0. 75	2. 0	1. 05	0. 75	0. 48
Choline·HCl	25. 0	—	0. 05	0. 125	1. 0	1. 0
Thiamine·HCl	1. 0	0. 5	0. 001	0. 0025	0. 1	0. 04–0. 0004
Riboflavine	0. 1	0. 05	0. 001	0. 0025	0. 1	0. 0004
Pantothenate	0. 1	0. 05	0. 001	0. 0025	0. 1	0. 0005
Nicotinamide	0. 1	—	0. 0025	0. 00625	—	0. 01–0. 001
Nicotinic acid	—	—	0. 0025	0. 00625	0. 5	—
Biotin	0. 002	0. 01	0. 001	0. 0025	0. 1	0. 001
Folic acid	0. 004	0. 01	0. 001	0. 0025	0. 1	0. 04–0. 004
m-Inositol·$2H_2O$	0. 1	—	0. 005	0. 0125	0. 5	No requirement
Vitamin B_{12}	0. 02	—	1. 0	1. 0	0. 0025	—

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We have retained in MB 752/1 all the amino acids of ML 192/2 (*29*). Work still in progress indicates that, in all probability, L-cystine (L-cysteine being a component of the medium), L-glutamic acid, L-aspartic acid, L-proline, and glycine are dispensable. Eagle found these acids (with the exception of L-cystine) and L-alanine, L-hydroxyproline, and L-serine, none of which was included in ML 192/2, to be inessential for strain L cells (*39*). However, Eagle included in his medium only L-cystine and not L-cysteine. In MB 752/1, L-cysteine is present at a slightly higher concentration than in ML 192/2 (9.0 instead of 7.78 mg./100 ml.) and this probably meets the whole requirement, in agreement with the findings of Morgan and Morton (*40*) for chicken fibroblasts. There is striking agreement on the amino acids qualitatively needed for cells of several species (*41*), *e.g.*, chick (*13*), monkey kidney (*41*, *43*), human U12-705 (*44*), and rat (Walker carcinosarcoma 256) (*45*), and those needed by the strain L mouse cell (*8*, *14*, *34*, *39*), though Haff and Swim (*16*) claim that RM3-56 rabbit fibroblasts need 14 amino acids (in the presence of 2% dialyzed serum). Definite requirements for strain L cells under our conconditions have been demonstrated, by elimination experiments, for L-cysteine, L-tyrosine, L-lysine, L-methionine, L-threonine, L-valine, L-arginine, L-histidine, L-leucine, L-isoleucine, and L-tryptophan. Phenylalanine demands further study as a possible alternative to tyrosine; meantime, both tyrosine and phenylalanine are included.

Particular attention has been focused on the basic amino acids, lysine and histidine. Increased concentrations of these 2 amino acids proved to be markedly beneficial, and L-histidine·HCl is now included at 15 mg./100 ml. and L-lysine·HCl at 24 mg./100 ml. A similar stimulation did not occur with increased arginine, though the concentration in MB 752/1 (7.5 mg./100 ml.) is already higher than the optimum (4.2 mg./100 ml.) which is a limiting factor for growth of strain L cells, under somewhat different conditions when a modified Eagle medium is used (*46*).

The importance of glutamine (*29*, *47*) has been confirmed and the concentration increased sevenfold (to 35 mg./100 ml.) over the former level.

Magnesium

The medium MB 752/1 contains an increased concentration of magnesium supplied as $MgSO_4$. A high Mg concentration promotes adhesion of the cells to the glass and somewhat enhances the growth of the cultures (*cf.* *48*).

Vitamins of the B Group

Choline.—The high concentration of choline (25 mg./100 ml.), which is optimal under our conditions, calls for comment. Brues *et al.* (*49*) found that neither chick fibroblasts nor tumor cells were inhibited by choline up to 500 mg. per 100 ml. When choline has been included in other tissue-culture media, it has usually been at physiological concentrations, *i.e.*, 0.1 to 0.2 mg. per 100 ml. (*4*, *10*, *11*, *19*) or slightly higher (*2*, *9*).

The rate of proliferation of our cultures increased progressively with an increase of choline up to 25 mg. per 100 ml.

Thiamine·HCl.—Concentrations of thiamine far above the physiological range (0.005 mg./100 ml.) have been commonly employed in tissue-culture media (*e.g.*, 23, see *50*). Our earlier medium ML 192/2 contains a larger amount of this vitamin (0.5 mg./100 ml.) than had hitherto been usual in synthetic media, and this amount has now been doubled. The concentrations in White's media (*2*, *9*, *10*) have also been progressively increasing (0.01, 0.1, 1.0 mg./100 ml.), though the amounts in other synthetic media remain in the range from 0.0004 to 0.04 mg./100 ml. (*4*, *7*, *19*, *51*). In order to detect a deficiency of thiamine, as well as riboflavine, pyridoxine, biotin, and folic acid, it was necessary to maintain the cultures for at least 1 week in deficient media (*cf.* also *48*, *51*).

Riboflavine.—This vitamin is now included at twice the concentration used before (*29*). This is far above physiological range and is now equal to the amount supplied by White (*2*).

Pyridoxine.—The concentration of pyridoxine has also been doubled.

Pantothenate.—Calcium pantothenate was also supplied at double the previous concentration. Inhibition occurred at concentrations above 0.1 mg. per 100 ml.

Biotin.—The concentration of biotin previously used (0.01 mg./100 ml.) was found to be too high. Reduction by one fifth (to 0.002 mg./100 ml.) improved growth. A long-term study of lower concentrations, and of media completely biotin-free, is in progress.

Folic acid.—A folic-acid deficiency, induced in 1 week on a folic-acid-free medium, can be reversed either by folic acid (pteroylglutamic acid) or by leucovorin (synthetic citrovorum factor). The optimum concentration of folic acid (0.05 mg./100 ml.) is 5 times the amount used in the peptone-containing media (*29*) and is well above the physiological level.

Nicotinic acid and nicotinamide.—In the presence of peptone, no requirement was found for nicotinamide. In peptone-free media, a rather high concentration (0.1 mg./100 ml.) of nicotinamide was effective in promoting proliferation. Mole for mole, nicotinic acid was slightly less active in this system.

Vitamin B_{12}.—Vitamin B_{12}, not included in our earlier media (*29*), has been added at a rather high dosage (0.02 mg./100 ml.) in MB 752/1. This is still, however, considerably lower than the concentration (1.0 mg./100 ml.) used by Healy *et al.* (*7*) and by Evans *et al.* (*4*). Vitamin B_{12} significantly improves the growth of the cultures. It remains to be seen whether lower concentrations would be equally effective, or whether Co ions alone could substitute for the vitamin.

Inositol.—Eagle *et al.* (*52*, *53*) have no requirement for inositol for strain L cells. This vitamin, included by Healy *et al.* (*7*), Evans *et al.* (*4*), and White (*2*), but not hitherto in our own media (*1*, *29*), has no evident short-term effect, and is included here on the basis of limited observations of improved appearance of fairly long-term cultures (several months). Longer experiments are needed to settle more precisely the qualitative

and quantitative contributions of inositol to the growth of the strain L cells.

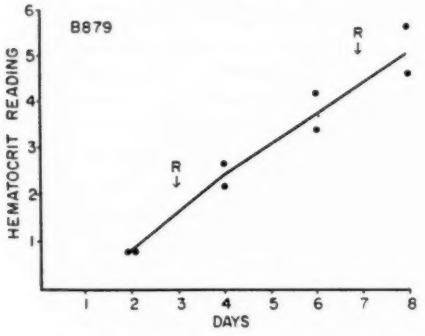
Growth of Strain L Cells in Medium MB 752/1

Stock cultures (fig. 1) from our subline maintained for over a year in the medium ML 838/1, a protein-free modification of ML 192/2, were used to initiate the subline of cells in MB 752/1 used for quantitative experiments on growth rate. The rate of proliferation of cultures of this subline in the fifth passage in MB 752/1 is illustrated in text-figure 1. The cell population increased approximately sixfold over a 6-day period, with twice weekly renewals of the medium. Text-figure 2 shows the rate of growth in the ninth passage, with a 7.5-fold increase in 8 days. Text-figure 3 relates to cultures in the 22d passage (after a total of 230 days in MB 752/1) and demonstrates an 8.3-fold increase in 8 days. The culture shown in figure 2 had been 10 days in the tenth passage in MB 752/1, and figures 3 and 4 show cultures at 4 days in the 21st and 7 days in the 22d passage. Two sublines of strain L in MB 752/1 have been maintained through over 30 passages (300 days), and no significant change in the rate of proliferation has been observed.

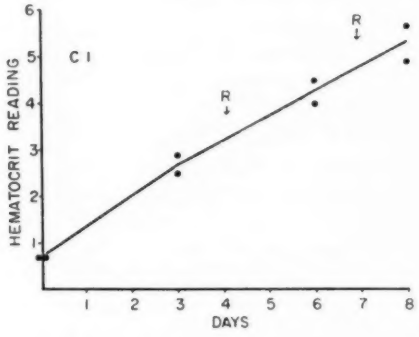
Cells from the subline initiated in August, 1958, directly from biological medium into MB 752/1 grew without difficulty in the synthetic medium. During the first 3 or 4 passages, the cells did not attach well to the glass but later—possibly by selection—attachment was as good as that with the older lines. The cells had a less confluent habit than those of the cells derived from our 5-year-old stock, even when well attached to the glass. After 12 passages in MB 752/1, they differed little in appearance from the other sublines in the same medium.

Discussion

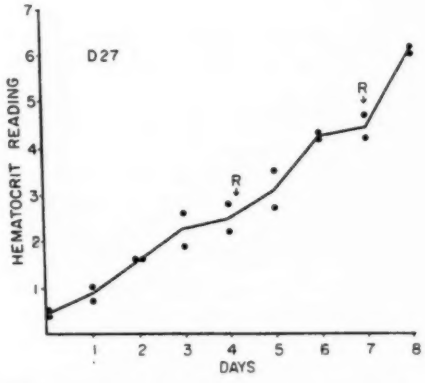
Biggers *et al.* (*54*), in a review published last year on growth factors for tissue cultures, rightly emphasize that ". . . the design of chemically defined media is still in its infancy. . . ." The demonstration that continuous proliferative growth of one cell strain is possible in a medium of considerable simplicity is possible evidence of some maturation, and effectively contraverts the recent statement of Graff and McCarty (*55*): "As everyone knows very well, metazoan cell culture differs from microbiological culture in that the former requires the inclusion of serum proteins in the medium." Not every component of MB 752/1 has been fully studied to establish optimum concentrations in relation to the rest of the solution, and further simplification is likely. Perhaps this medium is more interesting for the things that it does not contain, (*e.g.*, proteins and peptides), because they are found not to be required by the strain L sublines studied, than for the things it does contain, all of which are simple compounds which have been included in other tissue-culture media. Important *quantitative* changes have, however, also been introduced, notably in increasing the concentrations of glutamine, choline,



TEXT-FIGURE 1.—Rate of growth of strain L cells (fifth passage) in medium MB 752/1. R indicates nutrient renewal.



TEXT-FIGURE 2.—Rate of growth of strain L cells (ninth passage) in medium MB 752/1. R indicates nutrient renewal.



TEXT-FIGURE 3.—Rate of growth of strain L cells (22d passage) in medium MB 752/1. R indicates nutrient renewal.

thiamine, folic acid, and of the 2 basic amino acids, lysine and histidine. The concentration of biotin has been reduced, since a higher concentration has proved inhibitory to the growth of these cells under our conditions.

It has been stressed before (*50*, *56*) that concentrations of substances which are "physiological" in the context of the whole organism are by no means necessarily the optimal concentrations for a synthetic medium for isolated cells. The present medium demonstrates this point. It does not imitate in its composition any of the biological fluids, and, on the contrary, some of its components are present in amounts which are quite "unphysiological" in this sense. It is wise to bear in mind also that for many nutrilites there is, for a particular cell strain, no absolute "specific nutritional requirement." One substance may often be replaced by another, or a combination of several replaced by one. Interactions, antagonisms, and alternative metabolic pathways make nugatory the concept of "requirements" of, for example, those B vitamins whose function is to act as precursors of coenzymes. If the coenzymes themselves are furnished—or perhaps if the end product of the enzymatic process is supplied—the vitamin may not be required at all.

The rapid growth of sublines of strain L in MB 752/1 does not warrant the assumption that other cell strains will proliferate rapidly (or at all) in this mixture. On the contrary, such evidence as there is indicates that media must be designed to fit the needs of individual cell lines. No difficulty was experienced in achieving continuous proliferation in the synthetic medium MB 752/1 through more than 10 passages of a subline of strain L cells transferred directly from a high-protein, complex biological medium. No period of "adaptation" appeared to be necessary, that is, no retardation of growth rate occurred during early passages in the synthetic medium. There was a period of several passages during which the cells remained mostly in suspension and did not attach well to the glass, but thereafter the growth of this subline was very similar to that of the sublines more gradually adapted to the synthetic medium.

Strain L cells under good conditions rapidly form not only new cytoplasm and nuclei but also extracellular protein (*30*). One of the important factors in the capacity of this cell strain to survive and grow in protein-free media may be this ability to provide its own environmental protein, and this is most probably linked to the question of minimum inoculum size for the establishment of successful colonies (*33*, *57*, *58*). A culture with an inoculum of less than 10^5 cells per ml. of medium MB 752/1 either enters a prolonged lag phase before growth begins or shows active degeneration. An inoculum of 1.5×10^5 cells or more per ml. usually proliferates at once, doubling the cell population in 24 hours.

Solution MB 752/1 and cells from sublines of clone NCTC 929 (strain L) provide a system of rapidly proliferating cells in an environment initially completely defined and simple in composition. The cells, of course, alter the medium as soon as they come into contact with it; but this cell-nutrient system appears to be potentially useful for the study of the mechanisms of mitosis, of culture growth, and of cell metabolism.

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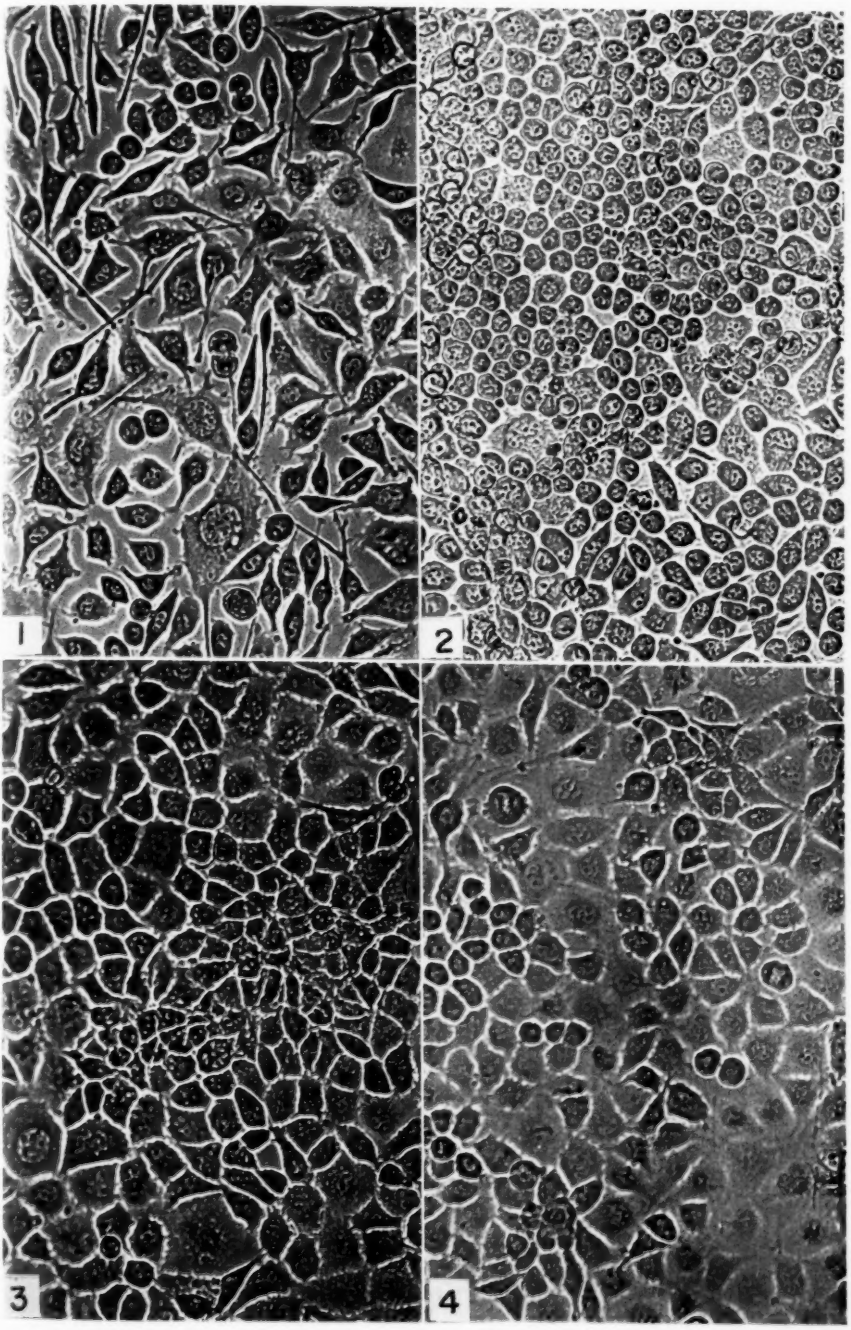
PLATE 94

FIGURE 1.—Strain L cells in protein-free, peptone-containing medium ML 838/1. Eight days after subculture. × 220

FIGURE 2.—Strain L cells in synthetic medium MB 752/1, tenth passage. Ten days after subculture. × 220

FIGURE 3.—Strain L cells in synthetic medium MB 752/1, 21st passage. Four days after subculture. × 220

FIGURE 4.—Strain L cells in synthetic medium MB 752/1, 22d passage. Seven days after subculture. × 220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Announcements

Cancer Publications

(Available from the National Cancer Institute)

Single copies of the following publications can be obtained free of charge from the Office of Information and Publications, National Cancer Institute, Bethesda 14, Maryland.

All items are published by the U.S. Government Printing Office, unless otherwise indicated. A price immediately following a publication indicates that it can be purchased in quantity from the Superintendent of Documents, U.S. Government Printing Office, Washington 25, D.C. Please use PHS publication number when ordering from the Superintendent of Documents.

For the general public:

- CAMERON, CHARLES S.: "The Cancer Quacks." 1957. 18 pp. Reprinted, with permission, from *The Truth About Cancer*, copyright, March 1956, by Prentice-Hall, Inc. A discussion of the serious problem of medical charlatans in the cancer field by the former Medical and Scientific Director of the American Cancer Society. The policy of the National Cancer Institute regarding investigation or evaluation of proposed cancer treatment is included in the Appendix. PHS Publication No. 559. Limited quantities available free of charge from the National Cancer Institute.

- GRANT, LESTER: "The Challenge of Cancer." 1950. 116 pp. Illustrated in color. A booklet telling the story of cancer research. Especially helpful to high school science classes. 70 cents each; 25% discount on orders of 100 or more. (See also, NATIONAL CANCER INSTITUTE, "Meeting the Challenge of Cancer" and "A Teaching Guide to *The Challenge of Cancer*," listed below.)

- JOHNSON, DALLAS: "The Facts About Cancer." 1957. 28 pp. A pamphlet giving general information about cancer, illustrated with charts and graphs. Order quantities from Public Affairs Pamphlets, 22 East 38th Street, New York 16, N.Y. 1 to 9 copies, 25 cents each; 10 to 99 copies, 20 cents each.

- NATIONAL CANCER INSTITUTE: "Breast Self-Examination." 1953. An illustrated fourfold leaflet in color showing the steps used in self-examination of the breasts. Available to women. PHS Publication No. 48. 10 cents each; $5 per 100 copies.

- NATIONAL CANCER INSTITUTE: "Cancer—What To Know, What To Do About It." 1958. A fourfold leaflet in color giving basic facts about cancer. PHS Publication No. 375. 5 cents each; $3.25 per 100 copies.

- NATIONAL CANCER INSTITUTE: "Meeting the Challenge of Cancer." 1955. 23 pp. A supplement to "The Challenge of Cancer" by Lester Grant (above). Presents some of the recent accomplishments in cancer research. PHS Publication No. 419. 15 cents each; 25% discount on orders of 100 or more.

- NATIONAL CANCER INSTITUTE: "The National Cancer Institute." Revised 1957. 23 pp. An illustrated brochure presenting the cancer problem and the history, organization, and program of the Institute. PHS Publication No. 458. 20 cents each; 25% discount on orders of 100 or more.

- NATIONAL CANCER INSTITUTE: "Reading on Cancer, An Annotated Bibliography." 1955. 16 pp. Lists books, pamphlets, reports, articles in popular periodicals and professional journals, and other pertinent material. PHS Publication No. 457. 15 cents each; 25% discount on orders of 100 or more.

▶ NATIONAL CANCER INSTITUTE: "A Teaching Guide to *The Challenge of Cancer*." 1950. 19 pp. A guide designed to help the science teacher use "The Challenge of Cancer" by Lester Grant (above) and the 16 mm. film, "Challenge: Science Against Cancer." 20 cents each; 25% discount on orders of 100 or more. (Film is available on loan from the National Cancer Institute.)

▶ NATIONAL CANCER INSTITUTE and AMERICAN CANCER SOCIETY: 20-page booklets with illustrations in color, giving important facts about a number of different types of cancer.

"Cancer of the Breast." Revised 1957. PHS Publication No. 576. 15 cents each; 25% discount on orders of 100 or more.

"Cancer of the Female Reproductive Organs." 1954. PHS Publication No. 376. 10 cents each; $6.25 per 100 copies.

"Cancer of the Digestive Tract." 1949. PHS Publication No. 474. 15 cents each; 25% discount on orders of 100 or more.

"Cancer of the Skin." 1954. PHS Publication No. 378. 10 cents each; $5 per 100 copies.

"Cancer of the Genito-urinary Tract." 1954. PHS Publication No. 377. 10 cents each; $5 per 100 copies.

"Cancer of the Mouth and Respiratory Tract." 1956. PHS Publication No. 427. 15 cents each; $8 per 100 copies.

▶ PUBLIC HEALTH SERVICE: "Leukemia." Revised 1957. A 2-page leaflet giving general information on leukemia, including the symptoms and treatment. PHS Publication No. 353. 5 cents each; $2 per 100 copies.

For professional readers:

▶ DORN, HAROLD F., and CUTLER, SIDNEY J.: "Morbidity from Cancer in the United States." 1959. 207 pp. A comprehensive and detailed analysis of data obtained from a series of cancer morbidity studies in 10 metropolitan areas made in 1938 and again in 1948 and 1949. Part I deals with variations in the incidence of cancer by age, sex, race, and geographic region; Part II compares the 10-city data with data from other sources and deals with trends in cancer morbidity during the decade between the two surveys. PHS Publication No. 590. $1 each; 25% discount on orders of 100 or more.

▶ DUNHAM, LUCIA, J., and DORN, HAROLD F.: "Techniques in Geographic Pathology of Cancer." 1954. 8 pp. Suggestions for a standardized technique for making a study of geographic pathology—sources of material, procedures for reporting material and for comparing one area with another, and ways to measure cancer frequency—are presented in this booklet. Single copies are available from the National Cancer Institute.

▶ GRISWOLD, M. H., WILDER, C. S., CUTLER, S. J., and POLLACK, E. S.: "Cancer in Connecticut, 1935–1951." 1955. 141 pp. A comprehensive volume of cancer statistics and information extracted from the Cancer Record Register maintained by the Connecticut State Department of Health. Reports the trend in cancer incidence in the total population of the State over a period of 17 years. Connecticut State Department of Health, Hartford, Conn. Limited quantities available from the National Cancer Institute.

▶ HAENSZEL, WILLIAM, MARCUS, SAMUEL C., AND ZIMMERER, EDMUND G.: "Cancer Morbidity in Urban and Rural Iowa." 1956. 85 pp. This study provided the first opportunity to collect and observe differences in morbidity data for both urban and rural populations using morbidity survey techniques developed by the National Cancer Institute. PHS Publication No. 462. 50 cents each; 25% discount on orders of 100 or more.

HAENSZEL, WILLIAM, SHIMKIN, MICHAEL B., and MILLER, HERMAN P.: "Tobacco Smoking Patterns in the United States." 1956. 111 pp. A detailed report of a survey on tobacco use made of a cross section of the population of the United States. Includes an addendum by Benno K. Milmore and Arthur G. Conover, "Tobacco Consumption in the United States, 1880–1955." PHS Publication No. 463. 60 cents each; 25% discount on orders of 100 or more.

NATIONAL CANCER INSTITUTE: A series of reports of cancer morbidity studies covering 10 major cities in the United States, based on data gathered in 1938 and again 10 years later.

"Cancer Illness Among Residents in Atlanta, Georgia." Cancer Morbidity Series 1. Sidney J. Cutler. 1950. 43 pp.

"Cancer Illness Among Residents of San Francisco and Alameda Counties, California." Cancer Morbidity Series 2. William Grodowitz. 1951. 46 pp.

"Cancer Illness Among Residents of New Orleans, Louisiana." Cancer Morbidity Series 3. Irving I. Warran. 1951. 52 pp.

"Cancer Illness Among Residents of Denver, Colorado." Cancer Morbidity Series 4. Samuel C. Marcus. 1951. 39 pp.

"Cancer Illness Among Residents of Pittsburgh, Pennsylvania." Cancer Morbidity Series 5. Sidney J. Cutler. 1951. 46 pp.

"Cancer Illness Among Residents of Chicago, Illinois." Cancer Morbidity Series 6. Sidney J. Cutler. 1952. 49 pp.

"Cancer Illness Among Residents of Dallas, Texas." Cancer Morbidity Series 7. Samuel C. Marcus. 1952. 46 pp.

"Cancer Illness Among Residents of Birmingham, Alabama." Cancer Morbidity Series 8. Samuel C. Marcus. 1952. 49 pp.

"Cancer Illness Among Residents of Detroit, Michigan." Cancer Morbidity Series 9. Sidney J. Cutler and John C. Rowan. 1952. 48 pp.

"Cancer Illness Among Residents of Philadelphia, Pennsylvania." Cancer Morbidity Series 10. Sidney J. Cutler and Samuel C. Marcus. 1952. 43 pp.

Limited quantities of these reports are available from the National Cancer Institute. (*See also* DORN, HAROLD F., and CUTLER, SIDNEY J., "Morbidity from Cancer in the United States," a summary report of the 10-city studies.)

NATIONAL CANCER INSTITUTE: "Cancer Services and Facilities in the United States." Revised 1954. 148 pp. A report arranged alphabetically by State of available cancer services and facilities, including basic cancer statistics, summaries of State cancer legislation, and the names and addresses of the official and voluntary organizations conducting State cancer prograns. Compiled by Ora Marshino. PHS Publication No. 14. 45 cents each; 25% discount on orders of 100 or more.

NATIONAL CANCER INSTITUTE, Biometry Branch: "The Extent of Cancer Illness in the United States." 1958. 23 pp. 31 questions and answers with accompanying charts and tables representing a distillation of some of the presently available evidence on cancer illness. Valuable to the physician, public health worker, and cancer educator. PHS Publication No. 547. 25 cents each; 25% discount on orders of 100 or more.

NATIONAL CANCER INSTITUTE: "Half of All Cancer Involves Sites Accessible to Direct Examination." 1954. A threefold leaflet in color giving statistics on cancer of accessible sites. (Facsimile of an exhibit for professional audiences.) PHS Publication No. 324. Limited quantities available from the National Cancer Institute.

NATIONAL CANCER INSTITUTE: "Highlights of Progress in Research on Cancer." 1958. 51 pp. Items of interest on program developments and research studies conducted and supported by the Institute, revised annually. PHS Publication No. 623. 25 cents each; 25% discount on orders of 100 or more.

▶ NATIONAL CANCER INSTITUTE: "Vaginal Cytology Aids Early Diagnosis of Unsuspected Cancer." Revised 1956. A fourfold leaflet in color illustrating the aspiration method of taking a vaginal smear. PHS Publication No. 448. 10 cents each; $5 per 100 copies. (A leaflet of the same title prepared especially for nurses is also available from the National Cancer Institute.)

▶ NATIONAL CANCER INSTITUTE and NEW YORK STATE DEPARTMENT OF HEALTH: "Cancer Control—A Manual for Public Health Officers." 1952. 90 pp. A manual designed to assist in the planning of a cancer control program. Illustrated with charts, graphs, photographs, and drawings. New York State Department of Health. Limited quantities available from the National Cancer Institute.

▶ NATIONAL CANCER INSTITUTE and NEW YORK STATE DEPARTMENT OF HEALTH: "Cancer Nursing—A Manual for Public Health Nurses." Revised 1955. 88 pp. This manual was prepared as a tool to assist the public health nurse in recognizing the early symptoms of cancer and to enrich her home visits, interviews, and teaching. Illustrated with charts, photographs, and drawings. New York State Department of Health. Limited quantities are available from the National Cancer Institute.

For Students of Cancer

Public Health Reports, monthly scientific journal, published by the Public Health Service, U.S. Department of Health, Education, and Welfare, calls your attention to the following recent papers, among others, of interest to students of cancer:

"Diagnostic and therapeutic X-radiation in an urban population," by Abraham M. Lilienfeld. January 1959, pp. 29–35.

"Some epidemiological aspects of cervical cancer," by Raymond F. Kaiser and Alexander G. Gilliam. April 1958, pp. 359–367.

"Follow-up study of 844 neoplasm suspects identified in a mass chest X-ray survey," by Dean C. McClure. April 1957, pp. 307–316.

"Life experience of cancer patients in Tennessee tumor clinics," by Sara Lou Hatcher. April 1957, pp. 348–354.

"Epidemiological tests of theories on lung cancer etiology," by William Haenszel. February 1956, pp. 163–172.

Public Health Reports may be purchased by subscription for $4.25 a year (plus $1.75 for foreign mailing). Send check, money order, or Government Printing Office coupons to: Superintendent of Documents, U.S. Government Printing Office, Washington 25, D.C.

The Gordon Research Conferences

The Gordon Research Conferences for 1959 will be held from June 15 to September 4 at Colby Junior College, New London, New Hampshire; New Hampton School, New Hampton, New Hampshire; and Kimball Union Academy, Meriden, New Hampshire.

The conferences were established to stimulate research in universities, research foundations, and industrial laboratories. This is achieved by informal meetings consisting of scheduled lectures and discussion groups. Meetings are held in the morning and evening, Monday through Friday, with the exception of Friday evening. The afternoons are available for discussion groups.

The purpose of the program is not to review the known fields of chemistry and physics but primarily to bring experts up to date on the latest developments, to analyze the significance of these developments, and to provoke suggestions concerning the underlying theories and profitable methods of approach for making new progress. In order to protect individual rights and to promote discussion, it is an established rule of each conference that no part of the information presented is to be used without

specific authorization of the individual making the contribution, whether in formal presentation or in discussion. Scientific publications are not prepared as emanating from the conferences.

Registration and Reservations: Requests for attendance at the conferences, or for additional information, should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From June 15 to September 4, 1959, mail should be addressed to Colby Junior College, New London, New Hampshire. Individuals interested in attending the conferences are requested to send their applications to the Director at least two months before the opening date of the conferences. *All applications must be submitted on the standard application form which may be obtained by writing to the office of the Director.* Attendance at each conference is limited to approximately 100 individuals.

The complete program of the conferences was published in Science, March 6, 1959. The following is an outline of the cancer program:

Frederik B. Bang, chairman — Philippe Shubik, vice-chairman

August 31

Epidemiology: *J. Clemmesen,* Danish Cancer Registry, Copenhagen, Denmark
Epidemiology of lung and bladder cancer.
A. Lilienfeld, Johns Hopkins University, Baltimore, Md.
Epidemiology of breast cancer
Virus aspects: *F. Bang,* chairman, Johns Hopkins University, Baltimore, Md.
M. Epstein, Bland-Sutton Institute, Middlesex Hospital, London, England
Electron microscopy; purification of Rous virus
W. Bernhard, Cancer Research Institute, Paris, France
Electron microscopy of tumors
R. Buffett and *F. Levinthal,* Childrens Cancer Research Foundation, Boston, Mass.
A survey of the leukemia and related tumor viruses in mice

September 1

Polyoma virus: *S. Stewart,* National Cancer Institute, National Institutes of Health, Bethesda, Md.
Studies on the antigenicity of SE polyoma virus and its relationship to other viruses
B. Eddy, Division of Biologics Standards, National Institutes of Health, Bethesda, Md.
Studies on the latency of SE polyoma virus
W. Rowe, J. Harley, and *R. Huebner,* National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.
Studies on polyoma virus
Comparative oncology: *H. Schlumberger,* chairman, University of Arkansas, Little Rock
A. Braun, Rockefeller Institute, New York, N.Y.
A physiological basis for the autonomous growth of three non-self-limiting neoplastic diseases of plants
H. Ratcliffe, University of Pennsylvania, Philadelphia, Penna.
Comparative aspects of spontaneous tumors

September 2

Chemotherapy: *A. Goldin* and *H. Venditti,* National Cancer Institute, National Institutes of Health, Bethesda, Md.
Quantitative evaluation of drugs in mouse tumors
J. Sholer and *L. Schmidt,* National Cancer Institute, National Institutes of Health, Bethesda, Md.
Quantitative evaluation of drugs in rat tumors

W. Kessler and *R. Jones, Jr.*, Division of Research Services, National Institutes of Health, Bethesda, Md.
Quantitative evaluation of drugs in rat leukemia
G. Zubrod, E. Marshall, Jr., and *P. Waalkes*, National Cancer Institute, National Institutes of Health, Bethesda, Md.
Quantitative evaluation of drugs in man and correlation with animal data
Biochemistry: *P. Shubik*, Chairman, Chicago Medical School, Chicago, Ill.
E. Racker, Public Health Institute, New York, N.Y.
Carbohydrate metabolism in tumors
H. Garland, Stanford Medical School, San Francisco, Calif.
Radiotherapy

September 3

DNA and tumors: *R. Herriott*, Johns Hopkins University, Baltimore, Md.
Biological properties of nucleic acids
A. Fjelde and P. Sorkin, Pasteur Institute, Paris, France
Production of mouse tumors by nucleic acid extracts
U. Heine, Duke University, Durham, N.C.
RNA extracts of Rous tumors
Tissue relationships: *P. Grant*, chairman, Johns Hopkins University, Baltimore, Md.
A. Moscona, University of Chicago, Chicago, Ill.
Cellular interactions in experimental histogenesis
B. Boving, Carnegie Institute of Embryology, Baltimore, Md.
Invasion mechanisms in placentation.

September 4

Immunology test: *J. Makeri*, Muhlenberg Hospital, Plainfield, N.J.
Cancer detection by immunological methods
Discussion led by *A. Osler*, Johns Hopkins University, Baltimore, Md.
E. Porter, Rockefeller Institute, New York, N.Y.
Electron microscopy of carcinogenesis

Erratum

Volume 21, Number 1, July 1958

Page 170, text-figure 5: In the paper entitled "Studies on Mouse Lymphomas. III. Behavior of Tumor- and Non-Tumor-Cell Populations During Growth of Three Ascites Lymphomas," by Shelton and Rice, the abscissa for Lymphoma #2 and for L1210 was omitted. For both Lymphoma #2 and L1210, the abscissa should *read* "10, 40, 80, and 120."

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